The Sertoli cell as the orchestra conductor of spermatogenesis: spermatogenic cells dance to the tune of testosterone

Fotios Dimitriadis,[1] Chara Tsiampali,[2] Nikolaos Chaliasos,[1] Panagiota Tsounapi,[3] Atsushi Takenaka,[3] Nikolaos Sofikitis[1]

[1]Laboratory of Molecular Urology and Genetics of Human Reproduction, Department of Urology, Ioannina University School of Medicine, Ioannina, Greece; [2]Department of Ophthalmology, Kilkis General Hospital, Kilkis, Greece; [3]Department of Urology, Tottori University School of Medicine, Yonago, Japan

ABSTRACT

Spermatogenesis is contingent upon hormones and growth factors acting through endocrine and paracrine pathways either *in vivo* or *in vitro*. Sertoli cells (SCs) furnish essential factors for the successful advancement of spermatogenesis and spermiogenesis. Moreover, receptors for follicle stimulating hormone (FSH) and testosterone, which are the main hormonal regulators of spermatogenesis, are identified on SCs. Testosterone, FSH and luteinizing hormone are known to determine the destiny of germ cells and in their absence germ cells undergo apoptosis. Bcl-2 family proteins determine one signaling pathway which seems to be crucial for the homeostasis of male gametes. In addition to paracrine signals, germ cell development also relies on signals generated by SCs via direct membrane contact. The regulatory peptide somatostatin has an important role in the regulation of the proliferation of the male germ cells. Activin A, follistatin and FSH control germ cell development. *In vitro* culture systems have provided initial evidence supporting the achievement of the completion of the first and second male meiotic division *in vitro*. This review article provides an overview of the literature regarding the hormonal pathways governing spermatogenesis and spermiogenesis.

Key words: Gonadotrophin, Growth factors, Hormones, Spermatogenesis, Spermatogonial stem cells, Spermiogenesis

INSIGHTS INTO SPERMATOGONIAL DIFFERENTIATION AND PROLIFERATION

Spermatogonia are settled on the basal membrane of the seminiferous tubule.[1,2] A small fraction of

Address for correspondence:
Fotios Dimitriadis, Laboratory of Molecular Urology and Genetics of Human Reproduction, Department of Urology, Ioannina University School of Medicine, Ioannina, Greece; Tel.: +30 2310411121; e-mail: helabio@yahoo.gr
Received:13-03-2015, Accepted:17-09-2015

their population operates as unipotent stem cells to bring forth exclusively mature spermatozoa. These unique stem cells, in contrast to other adult stem cells, present some characteristic features. As immediate descendants of primordial germ cells and principal components of the germline they are theoretically immortal. In studies using murine serial germ cell transplantation, spermatogonia were demonstrated as having an extremely long lifetime.[3,4] A large number of spermatozoa are required to preserve male fertility

and the key role of spermatogonia is to colonize the basal membrane of the seminiferous tubule at a critical stage when germ cells need to expand through mitosis prior to meiosis. In addition, spermatogonia must preserve the integrity of male DNA against internal damages such as accumulation of replication faults and external risks such as exposure to environmental mutagens. It is thus clear that spermatogonial stem cells (SSCs) represent key regulators of the control of the equilibrated evolutionary changes of the genetic material.

The rise of A_0 spermatogonia as the result of the resumed proliferation of gonocytes marks the start of spermatogenesis.[5] The spermatogenic process is a cyclic procedure, which in mice can be divided into 12 stages (I-XII).[6] In stage VIII, only a few A-aligned (A_{al}) spermatogonia are present, whereas A-single (A_s) and A-paired (A_{pr}) spermatogonia are predominant. As stage X starts over, A_s and A_{pr} spermatogonia begin to expand in such a way that the populations of A_s and A_{pr} spermatogonia remain relatively constant, whereas the population of A_{al} spermatogonia increases. Stage XII is followed by stage I, while during stages II and III germ cell expansion is arrested and the cells enter the G_1-G_0 phase. During the subsequent stages VII and VIII, without any further division, nearly all A_{al} spermatogonia that have been produced during the active proliferation period differentiate into A_1 spermatogonia. Next there follows the S phase and in stage IX A_1 spermatogonia differentiate into A_2 spermatogonia, after which five subsequent divisions give rise to A_3, A_4, I_n (intermediate spermatogonia), B spermatogonia and primary spermatocytes, respectively.

In total, nine to eleven mitotic divisions occur during spermatogonial development. Once the populations of A_4, I_n and B spermatogonia have decreased, the proliferation period continues to stage VII. Between A_4, I_n and B spermatogonia and A_s, A_{pr} and A_{al} spermatogonia there appears to be a feedback mechanism. This is evident from the fact that when the populations of A_4-B spermatogonia decrease to 50% of those in the normal testis, A_s, A_{pr} and A_{al} spermatogonia continue to proliferate beyond stage II.

An issue still under debate is whether the SSC divisions are symmetrical or asymmetrical. During symmetrical divisions one stem cell produces either two stem cells or two interconnected cells destined to differentiate (A_{pr}). On the other hand, when SSCs divide asymmetrically it results in a stem cell and a cell destined to produce A_{pr} spermatogonia. Thus, not all A_s spermatogonia are true stem cells. During the development of spermatogonia two differentiation steps appear to occur. First, A_s spermatogonia differentiate into A_{pr} spermatogonia. From then on, the germ cells consist of clones of cells interconnected through bridges which increase in size. The second step consists in the differentiation of A_{al} to A_1 spermatogonia which includes marked changes in cell behavior. In Drosophila, germ stem cells normally divide asymmetrically, generating one stem cell and one gonialblast. The latter starts the differentiation with spermatogonial transient-amplifying divisions.[7] A cluster of somatic cells at the testis apical tip, which is called a hub, represents the stem cell niche. The apical hub cells are responsible for the maintenance of stem cell identity through the expression of the unpaired signaling ligand, which activates the Janus kinase-signal transducers and activators of transcription (JAK/STAT) pathway within germline stem cells.[7] In the adult Drosophila, the JAK/STAT pathway is involved in stem cell renewal in the male germline[8,9] In the early stages of embryonic development, JAK/STAT signaling plays an important role in sex determination[10] and modulates embryonic segmentation through the control of the expression of the pair-rule genes even-skipped, runt and fushi tarazu.[11,12]

In humans, the germ cells can be classified as follows:[13-18] progenitor A_{dark}-spermatogonia (stem cells or reserve spermatogonia); progenitor A_{pale}-spermatogonia; committed A_{pale}-spermatogonia; B-spermatogonia; preleptotene (young primary spermatocytes), leptotene, zygotene, pachytene and diplotene spermatocytes; secondary spermatocytes; spermatids; and spermatozoa. The progenitor A_{dark}-spermatogonia divide sporadically, but with as yet unidentified stimulation, and generate progenitor A_{pale}-spermatogonia. Progenitor and committed A_{pale}-spermatogonia are of similar shape and morphology, but the latter give rise to B-spermatogonia.

Four consecutive processes characterize human spermatogenesis:[18-20]

1. Proliferation and differentiation of A_{dark}- and A_{pale}-

spermatogonia, production of B-spermatogonia from the division of committed A_{pale}-spermatogonia and formation of preleptotene spermatocytes from the division of B-spermatogonia.

2. The spermatocytes enter meiosis with the last synthesis of DNA in preleptotene spermatocytes and two meiotic divisions which gives rise to spermatids.

3. Spermiogenesis which involves the transformation of the spherical spermatid to a sperm-like mature spermatid.

4. Spermiation which involves the release of spermatozoon into the tubule lumen.
   All the previous processes have distinct requirements of modulatory molecules derived from the nearby Sertoli, peritubular and Leydig cells, as well as the vascular network.

Spermatogenesis in humans is different from the process in other common mammals such as bulls, mice, rabbits, rats or stallions. Obvious differences include the 3-dimensional organization of the seminiferous epithelium and the low number of sperm produced daily per gram of testis.[18]

Spermatogenesis in several species, and particularly in the human, has been the target of several research efforts worldwide. There are many unanswered questions in the spermatogenesis process. For example, there is no information concerning the exact time in human spermatogenesis that the A_{pale}-spermatogonia start cellular divisions; also unknown is the exact number of spermatogonia mitotic divisions resulting in the production of primary spermatocytes.

HORMONAL CONTROL AND SECRETORY ACTIVITY OF SERTOLI CELLS

Within the seminiferous tubules, Sertoli cells (SCs) represent the only somatic cells. Their role in exocrine testicular function appears to be crucial and this is emphasized by the fact that (a) 'germ cell-only testes' have never been reported, (b) the presence of SCs is required in the vast majority of *in vitro* culture systems for successful differentiation of germ cells and (c) the number of SCs is directly related to the population of germ cells sustained by the testes.[21] Each SC can support a defined number

of germ cells, limiting in this way the expansion of the spermatogonial population. This is evidenced by the fact that SCs form germ cell niches which allow a certain number of germ cells to reside in or repopulate the seminiferous tubules.[22] Additionally, in experimental studies in which the size of the testis and/or the spermatogenic output was manipulated by changing the number of SCs, there was a relatively constant ratio between SCs and spermatids before and after the manipulation.[23-25]

The contribution of SCs to spermatogenesis is based on the production of critical factors necessary for the successful development of spermatogonia up to the stage of the spermatozoon. Griswold[21] reported that glycoproteins secreted by SCs are crucial for spermatogenesis and could be divided into three main categories. First, SCs secret glycoproteins which facilitate the transport of ions and hormones or provide bioprotective functions, such as androgen-binding protein (ABP), transferrin and ceruloplasmin. Second, SCs provide both proteases and protease inhibitors with a role in tissue remodeling processes that occur, for instance, during spermiation or movement of preleptotene spermatocytes into the adluminal compartment of the seminiferous tubule. Third, SCs furnish glycoproteins that function as structural components of the basement membrane between the SCs and the peritubular cells. A specific transferrin system has developed to ensure that the tight SC junctions are circumvented so that the germ cells can receive a supply of ferritin produced by SCs. Moreover, SCs secrete vasoactive peptides and tachykinins which have been shown, *in vitro*, to induce the release of lactate and transferrin by SCs and also to stimulate aromatase activity by SCs.[26]

Furthermore, SCs secrete other glycoproteins that serve either as growth factors or paracrine factors, such as c-kit ligand (stem cell factor; SCF), the Mullerian duct inhibiting substance, inhibin and glial cell line-derived neurotrophic factor (GDNF). Both survival and development of spermatogonia were found to be stimulated by the action of SCF.[27] When spermatogenesis starts, SCs dramatically change the production of soluble SCF to membrane-bound SCF,[28] pointing to an important role for the SCF/c-kit system in spermatogonial differentiation. The c-kit-receptor and SCF also mediate SC-spermatocytes cellular adhesions, which

activity has been corroborated by studies showing that SCs from mice mutant for membrane-bound SCF are not able to bind spermatocytes.[29] Cadherins are also secreted by SCs; their function is the maintenance of the structure of testicular tissue and cell architecture and identity. Studies in the rat model demonstrated expression of mRNA coding for the three 'classical' cadherins, E-, P- and N-cadherin, in the developing rat testis.[30] Additionally, SCs produce peptides including prodynorphin and nutrients or metabolic intermediates.[21] The transfer of metabolic products, such as carbohydrates, amino acids, vitamins, lipids and metal ions,[31] from the SCs to the proliferating germ cells is only possible due to the tight relationship between SCs and germ cells; it is therefore essential that germ cells receive enough energy substrates.[32-34] Moreover, SCs deposit extracellular matrix components such as collagen and laminin, form specialized junctions and demonstrate a well-organized cytoskeleton significant for maintenance of the seminiferous epithelium.[31]

A good knowledge of the metabolism of SCs and the production of lactate, acetate and other metabolic factors that are essential for the normal occurrence of spermatogenesis is imperative.[35] It has long been postulated that close metabolic cooperation exists between SCs and developing germ cells, since developing germ cells rely on the lactate produced by SCs as metabolic fuel.[35] Indeed, in germ cells lactate is not only used as fuel but also presents antiapoptotic properties.[32] Meanwhile, SCs demonstrate some unique peculiarities in regard to carbohydrate metabolism. In fact, the majority of glucose is converted into lactate and not oxidized via the citric acid cycle,[34,36] a phenomenon known as "the Warburg effect", when cells (such as cancer cells) convert glucose to lactate even in the presence of sufficient and physiological oxygen levels to support mitochondrial oxidative phosphorylation.[37] This peculiar metabolic behavior of SCs is consistent with the ability of the more-developed germ cells, such as spermatids, to use lactate as an energy substrate rather than glucose.[34] Although the reasons why SCs preferentially export lactate for germ cells are not entirely elucidated,[38] it has been demonstrated that lactate production increases as the SCs differentiate during pubertal development.[39] The SC uptakes glucose through the action of glucose transporters, particularly glucose transporter-1 and

glucose transporter-3.[40] Once glucose enters the cells, it is catabolized by a series of reactions (glycolysis) to pyruvate.[40] Pyruvate is converted to lactate by the lactate dehydrogenase. SCs also actively produce acetate. Both substrates, lactate and acetate, are exported to the adluminal fluid by monocarboxylate transporters to support the development of germ cells.[40] The lactate produced by SCs is then used as fuel by the developing germ cells, this implying that lactate is more than a metabolic substrate and can control spermatogenic cell development *via* a complex pathway.[35] Thus, lactate production by SCs appears to be an excellent target for male fertility control.[35] In-depth scientific research into SCs metabolism has demonstrated hormonal regulation of the latter. Alterations in SCs metabolism result in impaired lactate delivery to developing germ cells that may compromise spermatogenesis.[41]

In order to ensure satisfactory lactate concentration in the microenvironment where germ cells develop, SCs have the ability to adapt their metabolism according to the needs of the spermatic cells.[42] Several studies have focused on the hormonal control of SCs metabolism and the mechanisms behind such hormone-related effects.[42-44] Rato et al showed that 5α-dihydrotestosterone (DHT) and 17β-estradiol (E$_2$) are key modulators of lactate production/export by SCs and that DHT is more capable of inhibiting the lactate production/export under their experimental conditions.[41] In that study, it was determined that sex steroid hormones have key roles that reach far beyond reproductive processes. It was also demonstrated that DHT modulates glucose consumption and lactate production in cultured rat SCs. Moreover, both DHT and E$_2$ decreased lactate dehydrogenase-chain A and monocarboxylate transporter-isoform 4 mRNA expression levels after 50h treatment.[41] Similarly, studies using primary cultures of human SCs reported that following E$_2$ and DHT treatment, lactate production by SCs was altered after 50h of culture.[43] Although the exact mechanism is not yet fully elucidated, recent evidence suggests that sex steroid hormones action in SCs metabolism is mediated through modulation in glycolysis-related transporters and enzymes, particularly at the transcriptional level.[45] DHT decreased glucose transporter-1 protein levels and increased LDH activity after 25h, providing evidence for a

role of this hormonal action in the regulation of SC metabolism.[45]

Furthermore, the androgen receptors (ARs) also play an important role in the regulation of testosterone levels. They are localized in the testicular somatic cells, including Leydig cells, peritubular myoid and blood vessel smooth muscle cells and also in SCs. By contrast, germ cells of the mature testis do not seem to require functional ARs. This fact indicates that androgens affect spermatogenesis indirectly through SCs, as these cells interact directly with developing germ cells and express functional ARs.[46,47] Moreover, when ARs are ablated in SCs, germ cell development stops at the spermatocyte stages[46] or early spermatid,[48] meaning that ARs present in the SCs play a crucial role in maintaining SCs ability to support normal spermatogenesis and that that SCs are the primary site that mediates androgen support of regular spermatogenesis.

The contribution of estrogens in the initiation and maintenance of testicular function and spermatogenesis is quite perplexing as estrogens participate in numerous physiologic functions of the male reproductive system including the hypothalamus-pituitary-testis axis, Leydig cells, SCs, germ cells and epididymis.[44] The concentration of estrogen is high in rete testis fluid,[49] while in the rat epididymis it is about 25 times higher than the estrogen levels measured in the plasma,[50] suggesting that estrogens have a key role in the regulation of spermatogenesis and in epididymal function. Furthermore, some studies confirmed that SCs are the major source of estrogens in immature individuals, whereas Leydig cells produce estrogens in adults.[51] Estrogens have the ability to disrupt the development of fetal Leydig cells and to enhance spermatogenesis by inhibiting apoptosis of the post-meiotic spermatogenic cells.[52,53] Also, estrogens affect proliferation and differentiation of gonocytes and spermatogonia and inhibit testosterone production by Leydig cells.[52,53] Moreover, estrogens regulate the function of mature spermatozoa and the incubation of human spermatozoa, while estrogens are known to stimulate sperm functions such as motility,[54] lactate production[55] and the metabolization of several substrates.[56] Conversely, estrogens can also induce apoptosis in the spermatogenic cell via the Fas ligand (FASL) pathway and the release of cytochrome c from mitochondria.[57]

Adjacent SCs form specialized interconnections in the form of junctions, creating the Sertoli/blood-testis barrier.[58] This barrier causes a physiological compartmentalization of the seminiferous epithelium into basal and apical (or adluminal) compartments and its proper functioning is required for the normal occurrence of spermatogenesis.[59] The physical and nutritional support of spermatogenesis is only one of the purposes of the blood-testis barrier. One other fundamental function of this barrier is the creation of an immune privilege status within the testis, repressing the immunological response to autoantigens residing within germ cells undergoing meiosis and also in developing spermatids during spermiogenesis.[60] Moreover, SCs transport water from the interstitial fluid to the lumen in order to use it as a vehicle for sperm transport from the testis to the epididymis.[61] Another important role of the blood-testis barrier is the regulation of the exchange of substances between the rete testis fluid and the lymph or plasma,[62] controlling the luminal fluid content where germ cells develop. Thus, it is understandable that a) several diseases, (such as diabetes mellitus)[63] which deregulate the dynamics and functioning of the blood-testis barrier or b) other extreme metabolic disorders (such as obesity) which deregulate the energy demands of the reproductive system,[64] result in male subfertility/infertility.[63]

The SC contributes to the development of the male reproductive potential via additional mechanisms. It has been reported that SCs play an essential role in the maintenance of peritubular myoid cells phenotype in fetal and neofetal life.[65] The proliferation of the adult population of Leydig cells and the maintenance of Leydig cells numbers in the adult testis was also reported to be stimulated and regulated by SCs action.[65] Leydig cells control testosterone levels and are also known to play a crucial role in spermatogenesis. Furthermore, recent reports emphasize that the interaction between SCs and Leydic cells may additionally serve as a molecular target to control male fertility. However, further research efforts are mandatory to elucidate these mechanisms and their relevance for male reproductive health.[35]

SCs possess receptors for both follicle stimulating hormone (FSH) and testosterone, which are the principal hormonal regulators of spermatogenesis. FSHβ knock-out mice are fertile in spite of a decrease

in testicular size and a 75% reduction in sperm numbers.[66] The combination of small testes and fertility in FSHβ deficient mice tends to suggest that FSH is necessary for testicular volume but may not be absolutely essential for spermatogenesis and normal fertility.[66] Similarly, mutations of the FSH receptor have been associated with variably severe reduction in sperm count, though fertility has been maintained.[67] In contrast to FSHβ knock-out male mice which have very low FSH levels, male FSHR knock-out mice have 3-fold elevated FSH levels.[68] Moreover, FSH alone or in synergic action with testosterone prevents germ cell loss or restores spermatogenesis quantitatively in hypophysectomized animals.[69] *Ex vivo* studies in human testicular tissue demonstrate the role of FSH and testosterone in the prevention of germ cell apoptotic death.[70,71] This evidence implies that both hormones function as germ cell survival regulators. The expression of Fas ligand in the testis is mainly localized in SCs, which underlines the key role of SCs in the regulation of the apoptotic mechanisms of germ cells.[72]

Normal SC secretory function is of paramount importance for the regulation of the spermatogenic process. A few studies during the last eight years have indicated that specific phosphodiesterase families are expressed in the testis and play a role in the regulation of SC secretory function in azoospermic men and in the regulation of Leydig cell secretory function in oligoasthenospermic men.[73] Inhibitors of phosphodiesterase 5 have been shown to improve focal advanced spermatogenesis in azoospermic men[74] and sperm counts in oligospermic men.[73] Furthermore, the phosphodiesterase 11 knock-out mouse is infertile.[75] The expression of different phosphodiesterase families in the SCs, Leydig cells, peritubular myoid cells and spermatogenic cells is highly selective, this depicted in Table 1.

An understanding of the molecular mechanisms and the biochemical pathways responsible for the control of the metabolic cooperation between SCs and developing germ cells may open up new insights into the subfertility/infertility conditions associated with several systemic diseases affecting male fertility, such as diabetes mellitus,[40] and may offer a basis for investigation into the development of new therapeutic strategies.

MECHANISMS REGULATING GERM CELL SURVIVAL AND DEATH

Apoptotic regulation in the human testis

The maintenance of normal architecture of the seminiferous tubules is accomplished by a dynamic equilibrium of germ cellular regeneration and elimination. There are eleven different types of cell survival mechanisms that have been reported. Ten cellular survival mechanisms are genetically programmed, while necrosis is the only cell death mechanism that is non-genetically programmed and occurs in pathological conditions.[76] However, only three of the genetically programmed cell death mechanisms, known to include caspase-independent apoptosis,[77] caspase-dependent apoptosis[78,79] and anoikis,[80] are described in the testis. Caspase-dependent apoptosis is the most common cellular death mechanism in the testis and is distinct from other forms of cell death because of its participation in caspase activation via pathways utilizing death receptors and/or BCL-2 family regulators.

Apoptosis has earned keen attention due to its involvement in a range of human testicular pathologies, such as cancer and other diseases.[81] Sinha Hikim et al[82] have not only provided strong evidence that germ cell death during normal human spermatogenesis occurs via apoptosis but they have also indicated the presence of ethnic differences in the inherent susceptibility of germ cells to apoptotic cell death. In fact, testicular samples obtained at autopsy from five Chinese and nine non-Hispanic Caucasian men were analyzed using a modified TUNEL technique and showed that the incidence of spermatogonial as well as spermatid apoptosis was higher in Chinese than in Caucasian men. This data could also explain the greater efficacy of testosterone-induced spermatogenic suppression in azoospermia observed in Asian compared to non-Asian men.[82] Moreover, apoptosis has been reported to be a possible mechanism of 2-methoxy acetic acid-induced spermatocyte death in cultured seminiferous tubules of middle-aged human donors or of spermatogonial death in pre-pubertal boys.[82] The researchers also furnished strong evidence implicating apoptosis in human germ cell processes. During normal spermatogenesis, apoptosis regulates the homeostasis of SCs to germ cell number by inducing programmed cell

Table 1. Expression of different phosphodiesterase families in the Sertoli cells, Leydig cells, peritubular myoid cells and spermatogenic cells

Type of cell			PDE	Species	Reference
Sertoli cells			PDE3	Rat	Swinnen et al[205]
			PDE4		Swinnen et al[205]
			PDE4B		Farooqui et al[206]
			PDE4D		Levallet et al[207]
Leydig cells			PDE11A	Human	D'Andrea et al[208]
			PDE4B	Rat	Farooqui et al[206]
			PDE5		Scipioni et al[209]
			PDE8A	Mouse	Vasta et al[210]
			PDE11		Baxendale et al[211]
Peritubular myoid cells			PDE5	Rat	Scipioni et al[209]
Germ cells	Spermatogonia		PDE11	Human	Baxendale et al[211]
			PDE11	Mouse	Baxendale et al[211]
	Spermatocytes		PDE11	Human	Baxendale et al[211]
	Developing spermatocytes		PDE3B	Rat	Degerman et al[212]
	Pachytene spermatocytes		PDE4A	Rat	Salanova et al[213]
			PDE1C		Yan et al[214]
			PDE4D		
	Spermatids		PDE11	Human	Baxendale et al[211]
			PDE11	Mouse	Baxendale et al[211]
			PDE4A	Rat	Farooqui et al[206]
		Round	PDE4A	Rat	Salanova et al[213]
			PDE4D		Farooqui et al[206]
		Round/Elongating	PDE1A	Mouse	Yan et al[214]
			PDE1C		
		Elongating	PDE4D	Rat	Salanova et al[213]
	Spermatozoa		PDE1	Human	Fisch et al[215]
			PDE1A		Aversa et al[216]
			PDE3A		Lefievre et al[217]
			PDE4		
			PDE5		
			PDE6	Mouse	Baxendale et al[218]
			PDE3B		
		Acrosomal region/ head	PDE4D	Mouse	Baxendale et al[218]
		Flagellum	PDE1A	Mouse	Baxendale et al[218]
			PDE4D		
			PDE10A		

death to eradicate germ cells that fail to replicate their DNA properly during cell division. Apoptosis can be induced by specific stimuli, such as deprivation of hormones, exposure to ionizing radiation, various chemotherapeutic drugs, cell injury and cell stress.

Not all degenerating germ cells display the classical morphology of apoptosis and therefore the exact incidence of adult male germ cell apoptosis remains unclear. Spermatogonia and round spermatids almost certainly follow apoptotic death, since they demonstrate many of the classical morphological and biochemical features of apoptosis, as for example labeling of nuclei by terminal deoxynucleotidal transferase and compaction of DNA at the nuclear margin.[83] Apoptotic round spermatids often degenerate *en masse* as multinucleated symplasts. During apoptosis, germ cells are either phagocytized by SCs or sloughed into the tubule lumen. However, the extent of spermatocyte and elongated spermatid apoptosis is less clear; some can be labeled as terminal deoxynucleotidal transferase and annexin V without showing the characteristic nuclear changes usually associated with apoptotic death, possibly due to the unusual DNA configuration and morphology of these cells.[83]

Whether germ cells survive or die is decided by a composite network of signals including paracrine signals such as SCF, leukemia inhibitory factor (LIF) and Desert Hedgehog (Dhh),[84] as well as endocrine direct or indirect signals such as pituitary gonadotrophins, estrogens and testosterone, among others.[53,85] In addition, germ cells respond to external signals and to their internal milieu by triggering intracellular signaling pathways that ultimately determine their destiny.

The mitochondrial or intrinsic pathway of apoptosis in the human

The intrinsic pathway, which involves members of BCL-2 family proteins that regulate the release of cytochrome c from the mitochondria into the cytosol, seems to be crucial for male germ cell homeostasis. Some members of this family promote cell survival (i.e. BCL-2, BCL-XL and BCL-W, among others), while others antagonize it [(e.g. Bcl-2-associated X protein (BAX), BAK and BIM, among others)]. In normal cells, BCL-2 family proteins are found in the outer membrane of the mitochondria, bound to the adaptor protein APAF1.[86] BCL-2L2 (formerly BCL-W) is a significant pro-survival member of the BCL-2 family[87] that takes part in apoptotic modulation by dimerizing with the proapoptotic factor BAX[88] whose expression is a feature of germ cell apoptosis *in vitro*. It is generally agreed that the ratio of proapoptotic to pro-survival BCL-2 family proteins is the critical determining factor of cell destiny. Apoptotic signals have been proposed as being involved in the regulation of antioxidant defences, resulting in enhanced sensitivity to reactive oxygen species and the induction of the mitochondrial permeability transition.[89] In turn, this causes the release of cytochrome c into the cytosol and the entrance of surplus BAX into the mitochondria through the open pores of the membrane.[90,91] Pro-caspase 9 and APAF1, together with cytochrome C, activate caspase 9, with subsequent activation of executioner caspases 3, 6 and 7. The activated executioner caspases are involved in the cleavage of intracellular proteins such as poly (ADP-ribose) polymerase, lamin, actin and gelsolin, leading to apoptosis.[90,91]

The pro-survival protein BCL-2L2 has a significant function in the regulation of the testicular germ cell population. The incidence of germ cell apoptosis in Bcl-2l2 knock-out mice increases dramatically between 2 and 4 weeks of age.[87] Other members of the BCL-2 family are expressed in the testis, but their exact role in spermatogenesis is still obscure (i.e. Bad, Bok, Bcm and Boo9, among others). There is evidence that spontaneous apoptosis occurs in male germ cell subpopulations in the human and that BCL-2 family proteins are distributed preferentially within distinct germ cell compartments.[92] It appears that these proteins play a specific role in the processes of cellular differentiation and maturation during the human spermatogenesis process. In humans, BCL-2 and BAK are mainly expressed in the compartments of spermatocytes and differentiating spermatids.[92]

Human male germ cells show a preferential expression of BCL-X at the stage of spermatogonium, whereas BAX is mainly expressed in the nuclei of round spermatids. BAD can be observed by immunochemistry in the acrosome region of several stages of human spermatids. On the other hand, MCL-1 staining does not demonstrate a particular pattern in the human testis. In the human testis, BCL-2L2, p53 and

p21 cannot be detected. The apoptotic promoter BAX preferential expression in human round spermatids may suggest that round spermatids may be especially prone to apoptosis when DNA is damaged. Expression of most of the intrinsic pathway constituents such as Bcl-2l2, Bax mRNAs and proteins,[88] APAF1[93] and cytochrome c[94] has been reported in early germ cell types including spermatogonia and spermatocytes as well as in SCs throughout development.

Aged men present a significantly lower apoptotic rate in spermatogonia in comparison with controls.[95] However, in the latter study,[95] the balance of spermatogonial proliferation and apoptosis showed no significant difference between the group of aged men and the control group. This could be one of the reasons explaining why the spermatogonial population in aged men is similar to that of controls. On the other hand, the apoptotic rate of primary spermatocytes in aged men is significantly elevated compared with younger controls, resulting in a reduction of the population of human primary spermatocytes per SCs in aged men. Furthermore, it has been demonstrated that the expression of BCL-XL is inversely related to the apoptotic rate in human primary spermatocytes, indicating that BCL-XL may contribute to the regulation of human primary spermatocyte apoptosis.[95]

The death receptor or extrinsic pathway of apoptosis in humans

The death receptor or extrinsic pathway represents another major pathway of caspase-dependent cell apoptosis. This pathway includes the cell surface death receptors called FAS receptors (FASRs) which have emerged as the key regulators. FAS is a transmembrane receptor protein and contains a 'death domain'. Among the apoptotic receptors comprising the tumor necrosis factor receptor superfamily, CD95/APO-1 (FAS) is the best characterized. FAS is abundantly expressed in various tissues, particularly in activated T and B cells, thymocytes, hepatocytes and heart tissue.[96] Of particular interest is the observation that FAS ligand (FASL, a cell surface molecule) is constitutively expressed by cells in immune privileged sites such as the testis[97] and the anterior chamber of the eye.[98,99] The binding of FAS protein to its ligand and then to FASR activates the apoptosis as a result of the recruitment of the intracellular adaptor molecule,

FAS-associated death domain, which binds several molecules of caspase 8 proenzyme, leading to proteolytic activation. Subsequently, activated initiator caspases cleave executioner caspases, resulting in apoptosis.[100] Both physiological and experimentally induced germ cell apoptosis has been shown to follow the activation of this pathway.[78] Pentikainen et al[101] have demonstrated that the FAS-FASL system regulates germ cell apoptosis in the human testis. In humans and in rodents, FAS has been identified in germ cells, spermatocytes and spermatids and FASL in SCs.[72,101,102] Antibodies antagonistic to FASL block human germ cell apoptosis *in vitro*.[101] On the other hand, neither FAS nor FASL is expressed in spermatogonia.[103]

The control of SCs-derived factors over the fate of male germ cells

Germ cells are dependent not only upon paracrine signals but also on signals derived from SCs through direct membrane contact. Membrane-bound SCF is expressed on precursors of SCs within the embryonic genital ridge and its receptor, the c-kit tyrosine kinase, is expressed on the surface of adjacent primordial germ cells. The first wave of spermatogenesis also requires the presence of SCF. In adults, c-kit is expressed on the surface of spermatogonia, while membrane-bound SCF is expressed on the corresponding basal regions of SCs. *In vivo* studies have demonstrated that the blockage of SCF/c-kit interaction in adults results in increased incidence of apoptosis in spermatogonia and spermatocytes. The c-kit gene is located at the white spotting (W) locus in the mouse and on chromosome 4 in the human,[104,105] whereas its ligand, SCF, has been identified as an analogue of the murine Steel (Sl) gene and in humans is located on chromosome 12 encoded by 9 exons.[106] Both in the adult and in the postnatal testis, c-kit is detected in the proliferating spermatogonia A1-A4 and is also present in interstitial somatic Leydig cells.[107,108] On the other hand, it appears that only SCs produce SCF in the testis.[109] The SCF/c-kit system is involved in different functions in the testis, including germ cell adhesion, cellular proliferation, cell migration and anti-apoptotic activities. In experimental studies, homozygous mutations of W and Sl loci resulted in the absence of functional production of c-kit or SCF, respectively, and were associated with the absence of germ cells in the post-

natal testis. These alterations of spermatogenesis are related to defects in primordial germ cells migration and/or induction of apoptosis.[110] Therefore, one of the key regulators of spermatogenesis seems to be the SCF/c-kit complex.

In addition, SCs may utilize the FAS system to control the destiny of germ cells. With regard to the human testis, the expression and the activity of FAS and FASL are a matter of debate. Besides the immunoregulative role of FASL in the testis, the FAS system has also been proposed as a key regulator of physiological germ cell apoptosis.[72,101,111,112] As discussed earlier, caspase inhibitors inhibit programmed human germ cell death, suggesting that FAS-associated human germ cell apoptosis is mediated via the caspase pathway.[78] Experimental studies have recorded an up-regulation of FAS in germ cells undergoing apoptosis after *in vivo* administration of SCs toxicants.[72] However, in human testis, the withdrawal of survival factors during an enhanced apoptotic process was not accompanied by up-regulation of the expression of FAS. This may point to the existence of additional pathways leading to enhanced apoptosis in the human testis. Alternatively, it has been hypothesized that FAS activation may be more efficient in unfavorable conditions, thus increasing the ability of the FAS-FASL complex to induce apoptosis in human germ cells.[101]

SC apoptotic signaling is a hormonally regulated process and a deregulation in SC apoptotic signaling will affect germ cells, compromising spermatogenesis. Dias et al hypothesized that the lack of insulin could lead to alterations in apoptotic signaling. Using cultures of rat SCs they examined the effect of insulin deprivation on several markers of apoptotic signaling. SCs cultured in insulin deprivation demonstrated a significant decrease in mRNA levels of p53, Bax, caspase-9 and caspase-3, followed by a significant increase of Bax and decrease of caspase-9 protein levels compared with control cultures.[113] Caspase-3 activity was also decreased in SCs cultured in insulin deprivation conditions. These results indicate that insulin deprivation decreases caspase-dependent apoptotic signaling in cultured rat SCs evidencing a possible mechanism by which lack of insulin can affect spermatogenesis and fertility.[113] Sex steroid hormones could also play a role in the regulation of

mitochondria-related pro-apoptotic factors. Simoes et al investigated the influence of E_2 and DHT on the apoptotic signaling pathways in immature rat SCs, evaluating the mRNA expression of p53, the pro-apoptotic Bcl2 family member Bax, caspase-3 and caspase-9 as well as the protein expression of caspase-9, Bax, the anti-apoptotic Bcl2 and AIF. They also evaluated caspase-3 activity and DNA fragmentation (TUNEL assay) as endpoint markers of apoptosis.[114] The researchers demonstrated that E_2 and DHT act as apoptotic signaling modulators in *in vitro* immature rat SCs. Both DHT and E_2 down-regulated p53, Bax, caspase-9 and caspase-3 mRNA levels. Importantly, DHT alone decreased the protein levels of apoptosis-inducing factor (a caspase-independent death effector released from mitochondria),[115] whereas E_2 alone down-regulated cleaved caspase-9 protein levels (regulated by the release of cytochrome c from the mitochondria and its binding to Apaf-1 to form the apoptosome complex).[116] This evidence may suggest that androgens and estrogens are capable of modulating independent pathways of apoptosis, regulated by different pro-apoptotic factors. Both DHT and E_2 were able to reduce caspase-3 activity and DNA fragmentation, clearly confirming the anti-apoptotic action of DHT and E_2.[114]

Hormonal regulation of germ cell apoptotic death

Reproductive hormones including FSH, luteinizing hormone (LH) and testosterone are known to influence the destiny of male germ cells. In their absence germ cells undergo apoptosis. In addition, within the testes, 1-2% of testosterone is converted to E_2 by the aromatase enzyme.[117] There is evidence for both beneficial and detrimental effects of E_2 on spermatogenesis in humans and in experimental studies.[53,118,119] Estrogen treatment, which is thought to mimic a gradual withdrawal of gonadotrophins, leads to apoptosis of all germ cells including elongated spermatids.[120] On the other hand, is has been demonstrated that E_2 acts as a germ cell survival factor in the human testis *in vitro*.[121] Therefore, it appears that E_2 may have mixed effects on spermatogenesis in experimental models wherein overmuch is detrimental but estrogen receptor signaling is required for the maintenance of normal spermatogenesis.[117]

Within the seminiferous tubules in men, apoptosis

is induced under serum-free conditions *in vitro*.[70] This apoptosis can be suppressed by administration of testosterone, implying that testosterone in the human male is a vital germ cell survival factor. However, the exact apoptotic pathway by which androgen withdrawal induces germ cell death remains elusive. BCL-XL and BCL-2 in the testis are altered following long-term anti-androgen therapy for prostate cancer.[122] Therefore, it is tempting to hypothesize that androgen deprivation changes the expression of the BCL family proteins in germ cells.

Somatostatin (SRIF) is a regulatory peptide hormone produced by neuroendocrine, inflammatory and immune cells in response to ions, nutrients, neuropeptides, neurotransmitters, thyroid and steroid hormones, growth factors and cytokines.[123] It plays a role in the control of the proliferation of male germ cells. Its biological functions are mediated by five receptors (sst1-sst5).[124] In clinical trials it has been demonstrated that the injection of SMS201995, an SRIF analogue, in healthy adult males is followed by a rapid (2h after the injection) rise in serum testosterone level. This enhanced testosterone secretion takes place without a simultaneous increase in LH production, which suggests that SRIF is able to modulate testosterone production at the testicular level.[125] The presence of SRIF and its receptors in human testes[126] clearly points to the existence of auto/paracrine loops controlling local testosterone secretion. Indeed, SRIF receptors sst3, sst4 and sst5 are expressed in human normal testicular tissue, while sst1 and sst2 are usually absent. In the rat testis the receptors sst1, 2 and 3 have been identified mainly in spermatocytes and SCs.[123,127,128] In addition, the accumulation of sst1-3 mRNAs at a high level in round spermatids at stages I-VII and their dramatic decrease at stage IX when round spermatids have already begun their elongating changes possibly point to some effects of SRIF and its receptors on spermiogenesis.[129] Further experimental studies have provided evidence for an inhibitory role of SRIF in the regulation of spermatogonial proliferation.[130] In the perinatal porcine testis, SRIF might exert its actions both directly on spermatogonia by preventing SCF-induced proliferation and indirectly by inhibiting SCF mRNA expression by SCs.[130]

The stimulating and challenging issue is now to discover the intracellular apoptotic regulators of germ cells and to unravel their regulation by a variety of death signals. The mechanisms by which several pro-apoptotic and anti-apoptotic genes modulate the process of spermatogenesis will offer new insights into the molecular components of the cellular survival/death machinery within the testis that determine the germ cell fate including their growth, division or death. This knowledge could provide potential applications in the assessment and management of male infertility.

THE EFFECT OF GROWTH FACTORS ON THE APOPTOTIC PATHWAYS IN THE TESTIS AND ON GONOCYTE PROLIFERATION AND DIFFERENTIATION

The presence of the tumor necrosis factor alpha-related apoptosis-inducing ligand (TRAIL) and its receptors has been demonstrated in different human testicular germ cell types.[131] Moreover, both TRAIL and two of its receptors, DR5/TRAIL-R2 and DcR2/TRAIL-R4, have been localized in Leydig cells. In addition, another receptor, namely DR4/TRAIL-R1, is seen in human peritubular cells and SCs. Most probably the TRAIL pathway is involved in the induction of apoptosis in the human testis.[131] The fate of germ cells is regulated by several paracrine signals. LIF promotes the survival of primordial germ cells in culture. Moreover, other factors including interleukin-4 (IL- 4), basic fibroblast growth factor (bFGF), a soluble form of SCF, and the bone morphogenetic protein-4 (BMP-4) also promote primordial germ cells survival *in vitro*. In contrast, other *in vitro* studies showed that transforming growth factor-β (TGF-β) promotes gonocyte apoptosis *in vitro*. LIF, bFGF and ciliary neurotrophic factor promote the survival of gonocytes co-cultured with SCs.[132-134] In adults, members of the BMP family promote germ cell survival *in vivo*. BMP-8A and BMP-8P appear to provide survival signals to spermatocytes. SCs secrete Dhh, another paracrine signal known to promote germ cell survival indirectly. GDNF, neurturin, persephin and artemin belong to the TGF-β superfamily.[135-138] GDNF mRNA has been detected in many tissues in addition to the brain and kidney, including the intestine, stomach, muscle, cartilage, lung and testis.[139] Expression of GDNF mRNA in the testis is associated with the

proliferation of SCs. In addition, GDNF contributes to the paracrine regulation of spermatogonial self-renewal and differentiation.[140]

The platelet-derived growth factor-A (PDGF-A) and PDGF-B genes encode A and B chains of PDGF and are located on human chromosome 7p and 22q, respectively.[141] Many cells produce PDGF, which exerts its effects on cells through receptor phosphorylation, resulting in cellular responses such as migration, proliferation, contraction and alteration of cellular metabolic activities, as for example matrix synthesis, cytokine production and lipoprotein uptake.[142] The PDGF-A gene, PDGF-B gene and the genes encoding the PDGF receptor alpha and beta subunits are expressed in the human fetal testis and this expression is enhanced in the adult testis, indicating a connection between the PDGF system and the start of spermatogenesis.[143] Since human Leydig cells express both the ligands and receptors of the PDGF system, it can be speculated that the ontogeny of this cell type is strongly determined by PDGF.[143]

The *in vitro* clonogenic method has been used to investigate the growth factors regulating mouse gonocyte proliferation and differentiation.[144] In the latter study, it was observed that epidermal growth factor (EGF) and TGF-β had no inhibitory effect on gonocyte-derived colony formation. Similarly, neither Mullerian inhibitory factor nor Leukemia Inhibitory Factor (LIF) demonstrated any effects on the proliferation of gonocytes. The authors showed that the growth of gonocytes *in vitro* was optimal in the presence of fetal calf serum.[144] Thus, it has been concluded that no specific growth factors (with the probable exception of PDGF[145]) are necessary for the growth of gonocytes *in vitro*.[144] However, other investigators[146] demonstrated that the addition of GDNF to a culture of mouse SSCs had a positive effect on SSC maintenance. Forced expression of GDNF in transgenic mouse testes resulted in the accumulation of undifferentiated spermatogonia *in vivo* without a change in SSC proliferation kinetics. This suggests that GDNF inhibits spermatogonial differentiation.[140] Hasthorpe[145] evaluated the effect of SCF in the differentiation of spermatogonia and demonstrated that SCF did not exert any effect on mouse type A spermatogonia colony-forming cells. This indicates that more highly differentiated spermatogonia represent

the target-cellular population of SCF. The majority of gonocytes expresses c-kit mRNA but fails to respond to SCF, pointing to a non-functional receptor. PDGF seems to stimulate the proliferation of gonocytes, but not the proliferation of type A spermatogonia recovered from 15-day-old animals.[145]

Growth factors are key regulators of the fate and lineage determination of undifferentiated spermatogonia. A substantial subpopulation of them has been identified and their role has been slowly unraveled during the last few years. However, important scientific work is still pending in order to elucidate their effects and utilize the acquired knowledge in the development of treatments for male factor infertility.

HORMONAL REGULATION OF GERM CELLS

Hormonal regulation of gonocyte proliferation and differentiation

The addition of activin to a gonocyte culture system rules against any antagonistic effect of SCs (which produce inhibin-bA subunit) on gonocyte proliferation. However, although Hasthorpe[145] observed that activin increased gonocyte colony formation, very little effect on spermatogonia cells was demonstrated in this study.[145] On the other hand, other investigators noticed that the addition of activin to culture systems significantly decreased the population of SSCs,[146] suggesting that the stimulation of spermatogonial proliferation by activin may be exerted on more advanced spermatogonia rather than on SSCs. The inconsistency of the findings may be assigned to the dissimilar methods employed for the selection of a spermatogonial population for culture. In the first study, Hasthorpe[145] used the *in vitro* clonogenic method, whereas in the second study Nagano et al[146] applied a two-step enzymatic digestion method on cryptorchid testes to extract spermatogonia as had been described earlier by Ogawa et al.[147] This particular method results in the recovery of a heterogeneous population of germ cells which also includes testicular somatic cells within the population of the recovered cells. These somatic cells might have had a negative effect on the proliferation of SSCs in the study by Nagano et al.[146] Moreover, the use of feeder layers in the study by Nagano et al[146] might also

have had a negative effect on the proliferation of the SSC population. Generally, the *in vitro* attempts to improve the viability and differentiation of cultured gonocytes by adding several growth factors to the basic medium have not yielded any clear results to date. Activin-A, follistatin and FSH appear to play a role in germ cell maturation during the period in which gonocytes resume mitosis to form the SSCs and differentiating germ cell populations.[148] It has been proposed that germ cells have the potential to regulate their own development initially through the production of endogenous activin-A.[148] SCs were observed to produce the activin/inhibin-bA subunit, the inhibin-A subunit and follistatin, demonstrating that these cells play a key role in the regulation of germ cell maturation and development.[148] The authors used 1- and 3-day-long cultures of 3-day-old rat testicular fragments and observed that treatment with activin-A produced a significantly higher ratio of germ cells to SCs, whereas treatment with follistatin and FSH enhanced the population of spermatogonia.[148] Hence, it appears that locally produced activin can stimulate gonocyte proliferation immediately after birth in the rat testis.[148] Therefore, it is possible that activin and follistatin may play a critical role in the development of gonocytes into spermatogonia.

Other investigators demonstrated that FSH has an indirect effect on the gonocytes by inducing SCs expression of follistatin and inhibin.[149] It appears that the maturation of gonocytes to form spermatogonia could result from the combined action of follistatin and inhibin as activin antagonists, with FSH as the stimulus for inhibin production, thus producing effects on both germ cells and SCs that control germ cell maturation. *In vitro* experiments furnished evidence that FSH and activin stimulate SC proliferation during early postnatal testis development.[150,151] Furthermore, the EGF receptor is functional in spermatogonia and it has been proposed that EGF suppresses testicular germ cell differentiation. Haneji et al[152] have established that EGF inhibits the proliferation of adult mouse type A spermatogonia stimulated by FSH, whereas Wahab-Wahlgren et al[153] observed a stimulatory effect of EGF on spermatogonial proliferation in adult rat seminiferous tubules *in vitro*, with a possible important role in the paracrine regulation of spermatogenesis.

Hormonal regulation of advanced spermatogonial development

In vitro culture systems have provided evidence that spermatogonia in an advanced stage of differentiation have different regulatory mechanisms that control their fate in comparison with the mechanisms regulating gonocyte proliferation.[122] Thus, both SCF and its receptor c-kit have a key role in relatively advanced spermatogonial development. It has been shown that SCF acts as a mitogen and survival factor for spermatogonia type A cultured in a Potassium Simplex Optimized Medium, i.e. a potassium-rich medium.[154] In the same study, granulocyte macrophage colony stimulating factor also enhanced the survival of porcine type A spermatogonial cells.

Insulin-like growth factor-I and -II (IGF-I and IGF-II, respectively) as well as insulin have been shown to promote spermatogonial differentiation into primary spermatocytes.[155] These findings corroborate the data furnished by Tajima et al[156] who have demonstrated, *in vitro,* that IGF-I and TGF-α enhances the differentiation of mouse type A spermatogonia in organ culture. On the other hand, neither PDGF nor fibroblast growth factor (FGF) has the above stimulatory effect. Other investigators, employing a Vero cell conditioned medium rich in growth factors and ILs, showed that FSH inhibits human spermatogonia degeneration and stimulates meiosis entry, being further potentiated by testosterone.[157]

In another series of studies, Tesarik et al[158,159] demonstrated *in vitro* that high concentrations of FSH are a requirement for the completion of meiosis and spermiogenesis. In the latter studies, cultured round spermatids, after the addition of high concentrations of FSH into the culture medium, underwent nuclear changes similar to those taking place during the normal spermiogenesis process with morphological characteristics including nuclear condensation, peripheral migration and protrusion. It should be stressed that in an *in vitro* culture system the high concentration of FSH (25 IU/l) is mandatory to obtain alterations in spermatid morphology.

Hormonal regulation of spermiogenesis

Spermiogenesis implies the maturation and differentiation of the early haploid male gamete to a mature spermatozoon. During the spermiogenetic

process, modifications occur in the entire male gamete including nuclear proteins, cellular size, cellular shape, the position and size of proacrosomal granules and the localization of the centrioles. This fascinating transforming process that converts a round immotile haploid gamete to an elongated cell with potential to move is regulated by a complex of factors/mechanisms. Since immunoreactive ABP has been detected in SCs processes that surround the elongated spermatids, it may be suggested that ABP plays a role in spermiogenesis.[160] ABP has a high affinity for androgens, probably contributing to the generation of high androgen concentrations in the vicinity of certain meiotic germ cells.

The exact role that androgens play in spermiogenesis and spermiation is not clear, but the fact that it is crucial can be deduced from most genetically-modified models of AR ablation in which the germ cells do not reach the spermatid stage.[161] However, the process of spermiogenesis can be studied in androgen-withdrawal models and results indicate that the adhesion between SCs and spermatids is androgen-dependent, since androgen action is required to prevent the retention and phagocytosis of mature, elongated spermatids and the premature release of round spermatids.[48,162] Experimental studies[163,164] have suggested that following withdrawal of intratesticular testosterone, round spermatids are unable to proceed through the transition between steps 7 and 8 of spermiogenesis and therefore cannot complete the elongation process. This effect could be the result of the loss of the adhesions of the spermatids to the sustentacular SCs.[165] Complementary studies in the rat experimental model have demonstrated released step 8 round spermatids within the epididymal lumen of animals with low intratesticular testosterone profiles.[164] It has been shown that decreased intratesticular testosterone concentration leads to apoptosis of germ cells.[166] In addition, a consequence of reduction in intratesticular testosterone is the loss of adhesion of round spermatids to the SCs, with subsequent slough into the lumen of the seminiferous tubules and occasionally phagocytization by SCs. The Bcl-2-modifying factor (Bmf) is likely to play a significant role in germ cell death in response to diminished intratesticular testosterone profiles.[80] Bmf has been detected in the subacrosomal space of spermatids of steps 4-16 of the spermioge-

netic process. The detection of Bmf in this region is not surprising since Bmf is normally sequestered to the actin cytoskeleton via its conserved dynein light chain-binding domain.[167] The actin polymers in the subacrosomal space have been shown to disappear in the late stages of spermiogenesis (step 19) just before the release of mature spermatozoa from the SCs.[80,168] Show et al[80] have provided strong evidence supporting the absence of the Bmf protein from the subacrosomal space near the end of spermiogenesis (step 16), which implies that Bmf is degraded just before the spermatid is released from the seminiferous epithelium.

There are studies indicating that testosterone together with FSH promotes spermiogenesis by promoting adhesion of round spermatids to sustentacular SCs.[169]

Several investigations demonstrate that some patients with spermatogenic arrest at the primary spermatocyte stage or Sertoli cell-only syndrome may have rare foci of arrested round spermatids somewhere in the testicle.[170-179] The inability of the round spermatid to undergo elongation in the human is accompanied by poor results in assisted reproductive technology programs using ooplasmic injections of round spermatids for the alleviation of male infertility.[180] Amer et al[175] used the term 'complete spermiogenesis failure' for men in whom the most advanced germ cell present in the therapeutic testicular biopsy material is the round spermatid and the term 'incomplete spermiogenesis failure' for non-obstructed azoospermic men with a very limited number of elongated spermatids in their therapeutic testicular biopsy material. It appears that in some men with spermatogenic arrest at the primary spermatocyte stage, a number of germ cells can break the barrier of the premeiotic block and differentiate up to the stage of round or elongating spermatids.[175,177,180] Steps 7 and 8 in the classification of the 16 steps of the rodent spermiogenesis[180] represent the beginning of the elongation of the round spermatid. The elongation of the round early haploid male gamete is accompanied by an increase in the reproductive capacity of this early haploid male gamete.[180] Thus, the beginning of the elongation in the round early haploid male gamete is of paramount importance because it allows its further differentiation *in vivo* and the subsequent development of elongating spermatid, elongated spermatid or spermatozoon within the

seminiferous tubule (otherwise there is arrest at the round spermatid stage). In addition, elongation of the round spermatid is accompanied by higher fertilization, pregnancy rates and live birth rates in assisted reproductive technology programs using ooplasmic injections of immature haploid male gametes.[180] Several biochemical mechanisms may be responsible for the inability of the round spermatid to undergo the elongation process in rodents. O'Donnell and co-workers have shown that suppression of intratesticular testosterone content may be one of the mechanisms.[163] Additional studies are necessary to clarify whether an intratesticular testosterone concentration below a specific threshold causes failure of elongation of round spermatids. If this hypothesis is correct, testicular pathophysiologies affecting optimal intratesticular testosterone content may lead to complete spermiogenetic failure. It should be emphasized that varicocele, the most frequent cause of male infertility, known to occasionally cause azoospermia, is accompanied by a reduced intratesticular testosterone concentration.[181]

There are differences in specific early events of the spermatogenesis process mainly prior to the first meiotic division in the human, the monkey and rodents (see for review Sofikitis et al[182]). However in most of the species, the transformation of the round spermatid to a mature spermatozoon is a common multistep process involving a gradual elongation of the early haploid male gamete, development of the male gamete tail and a protrusion of the male gamete nucleus in proximity with the plasma membrane.[182]

The synergic action of FSH and testosterone is mainly demonstrated in spermiation. The suppression of both hormones resulted in further induction of spermiation failure to a percentage equal to 90% compared with their individual separate effects, which were about 15%.[183] The latter effects are likely mediated by β_1-integrin in an integrin-associated kinase-independent mechanism.[184] Indeed, Beardsley and O'Donnell[184] aimed to determine the effects of acute testosterone and FSH suppression on the morphological and ultrastructural events associated with spermiation. In addition, they studied the localization of cell adhesion molecules (integrins and cadherins) and associated molecules (β-catenin and integrin-linked kinase) during hormone suppression-mediated spermiation failure in an experimental animal

model. β_1-integrin and one of its associated kinases, integrin-linked kinase, are candidates for mediating/regulating adhesion between the spermatid and Sertoli cell during spermiation.[185] It has been concluded that spermiation failure after testosterone and FSH suppression is caused by a dysfunction in the final disengagement of spermatids from the Sertoli cell rather than by a dysfunction in earlier events, such as ectoplasmic specialization removal, during spermiation. Immunohistochemical data suggested that β_1-integrin, not in association with integrin-linked kinase, plays a role in such normal disengagement by a "loss of adhesion" mechanism. During spermiation failure, this β_1-integrin-mediated loss of adhesion is perturbed, resulting in retained spermatids with associated β_1-integrin immunostaining.[184]

The role of FSH alone in the control of spermiogenesis has been a controversial issue. In the international literature there are studies supporting the notion that FSH may have a stimulatory effect in the early events in spermatogenesis including spermatogonial proliferation and meiosis. However, testosterone alone is capable of sustaining complete spermatid differentiation.[186,187] Studies by Baccetti et al[188] showed that exogenous FSH administration with or without simultaneous administration of human chorionic gonadotrophin in infertile men improved sperm counts. Moreover, the administration of FSH had a beneficial effect on sperm cytostructural parameters.[188] Other experimental studies[189] have demonstrated that there is an increase in propidium iodide stainability of elongated spermatids and an increased sperm head size in FSH receptor knock-out mice. In light of these findings, it appears likely that a disturbance takes place in the normal replacement of histones by protamines throughout spermiogenesis, leading to inadequate condensation of spermatid nuclei in FSH receptor knock-out mice.[189] FSH provides SCs with the ability to effectively bind round spermatids. In addition, studies in humans showed that FSH stimulates meiosis II and round spermatid flagellum extrusion, whereas testosterone potentiates FSH action and stimulates late spermatid differentiation.[122,157]

Studies in diabetic patients tend to point to a role of insulin in the spermiogenetic process.[190] In addition, insulin gene family members have been identified in round spermatids of human and rat testes, a fact that

provides additional evidence for a role of insulin and IGF in spermiogenesis.[191] Prolactin is another hormone that may play a potential role in spermiogenesis. Prolactin receptor expression has been detected in round and elongating spermatids of intact sheep testes.[192] However, other experimental studies in rat early round spermatids did not detect prolactin receptor mRNA expression,[193] indicating species-dependent differences in the hormones regulating spermiogenesis. The identification of type I and type II activin receptors in round spermatids suggest that activins may play a role in early haploid male germ cells.[194] Inhibin B in the testicular tissue of adult men is possibly a joint product of SCs and germ cells (including the stages from pachytene spermatocytes to early spermatids[195]). Other studies employing immunohistochemistry and *in situ* hybridization techniques[196] demonstrated that the inhibin-bB subunit is detectable in germ cells from the pachytene spermatocytes up to round spermatids, but not in SCs. The actions of activin may be regulated by actions of follistatin, which has been shown to be produced by SCs, spermatogonia, primary spermatocytes and round spermatids.[197]

Successful spermatogenesis is the cornerstone of male fertility. As SCs support and nourish the developing germ cells, they certainly merit particular attention in the process of spermatogenesis. SCs have multiple roles in germ cell development including physical support, immunoprotection and supply of nutrients and other factors. On the other hand, germ cells have specific metabolic needs, which convert during their transformation into spermatozoa, rendering them dependent on the nourishment provided by SCs. SCs employ different substrates, such as glucose and fatty acids, and pathways to accomplish their metabolic demands, as well as those of developing germ cells.

The characterization of SCs' energy metabolism, the preferred substrates, the metabolic sub-products produced and the mechanism of adaptation to different conditions[44] will offer new therapeutic approaches against several infertility-related disorders.

THE ROLE OF THE SC AS A POLICEMAN IN A HIGHWAY REGULATING THE TRAFFIC OF SPERMATOGONIA CELLS WITHIN THE SEMINIFEROUS TUBULUS

Table 2 demonstrates that donor mouse, rat, hamster or human spermatogenic cells transplanted into the testes of recipient immunodeficient xenogeneic animals colonized the recipient seminiferous tubules and initiated donor spermatogenesis within the recipients. It appears that the recipient species SC is able to support donor species spermatogenesis (both meiosis and complete spermiogenesis) within the recipient seminiferous tubules in the studies cited in Table 2. In these experiments, the initiation and maintenance of donor spermatogenesis and spermiogenesis within the recipient testes post-donor germ cell transplantation seems to be inconsistent with the fact that the donor spermatogonia have been injected and remained within the lumen of the seminiferous tubules. It appears that donor spermatogonia have moved from the recipient adluminal to the basal compartment of the tubules. Such cellular movement does not occur naturally in adult mammalian species. The mechanisms regulating the translocation of the injected donor spermatogonia from the lumen towards the basal surface of the recipient tubule cannot be easily explained. In contrast, the success of these experiments suggests very emphatically that the recipient Sertoli cell can regulate translocation of recipient and donor germ cells, respectively, either from the basement mem-

Table 2. The Sertoli cell regulates both donor and xenogeneic recipient spermatogenic cell meiosis and spermiogenesis within the same recipient seminiferous tubule.

Donor and recipient species	Authors
Rat spermatogenesis within mouse testis	Clouthier et al, Franca et al[219, 220]
Hamster spermatogenesis within rat testis	Sofikitis et al[99]
Hamster spermatogenesis within mouse testis	Sofikitis et al, Ogawa et al[99, 221]
Human spermatogenesis within mouse or rat testis	Sofikitis et al[222]

Donor rat spermatogonia had been collected from healthy animals;[219,220] Donor hamster spermatogonia had been recovered either from healthy animals or cryptorchid animals;[99,221] Donor human spermatogonia had been collected from non-obstructed azoospermic men.[222]

brane to the lumen or from the lumen to the basement membrane. Thus, the SC acts as a policeman in a highway regulating the traffic in both directions (i.e. from the lumen of the seminiferous tubule to the basement membrane and from the basement membrane to the lumen). In addition, given that hamster spermatogenesis lasts 35 days and rat spermatogenesis lasts 58 days, it appears that the production of both testicular rat spermatozoa and hamster spermatozoa within the same testis, after transplantation of hamster spermatogonia into immunodeficient recipient rats, is consistent with the hypothesis that the SC regulates the speed of spermatogenesis according to messages that it receives from the respective germ cells (either donor or recipients).[99]

GENOMIC IMPRINTING IN THE CELLULAR PRODUCTS OF MALE MEIOSIS AND SPERMIOGENESIS

The great majority of genes are expressed equally by the two parental alleles. However, a small subgroup of mammalian genes is differentially expressed depending on whether they have been inherited from the mother or the father. The process which differentially marks the DNA in the parental gametes is termed genomic imprinting. Genes whose expression is inhibited after passage through the mother's germline are called maternally imprinted. On the other hand, genes whose expression is inhibited when transmitted by fathers are called paternally imprinted.

Several imprinted genes have been characterized.[198] A few studies have shown that imprinted genes regulate the development of the embryo/fetus. Additionally, it has been proposed that DNA methylation maintains the imprinting of some genes. Abnormalities in genomic imprinting are associated with genetic diseases. Prader-Willi syndrome and Angelman syndrome are two examples of abnormal functional imprinting. It should be emphasized that abnormal functional imprinting is implicated in tumorigenesis.

Previous studies[199-201] have indicated that genomic imprinting is complete at the rabbit round spermatid stage and the mouse primary spermatocyte stage. However, additional studies are necessary in the human to confirm that genomic imprinting has been completed at the round spermatid stage. If genomic imprinting is incomplete in subpopulations of men with late maturation arrest or hypospermatogenesis, abnormalities may not become manifest in early embryonic development but they may be detectable in the fetus or during postnatal life. A question of major clinical importance is whether genomic imprinting has been completed at the human round spermatid stage. To attempt to answer this question, the imprinting of a gene should conceptually be divided into three stages: (i) erasure of the previous imprint, (ii) re-imprinting and (iii) consolidation of the new imprint. Several studies have suggested that erasure of the previous imprint occurs prior to meiosis and that re-establishment of the new imprint begins prior to the pachytene stage of meiosis (see for review Tycko[198]). On the other hand, the fact that DNA methyltransferase enzyme is present in spermatids may be an argument against the thesis that genomic imprinting is complete at the round spermatid stage. However, it should be emphasized that waves of DNA methylation have been demonstrated during early embryonic development, the blastocyst stage and the time of implantation.[202] These observations tend to indicate that even if genomic imprinting is not complete at the round spermatid stage, genomic imprinting may be completed after the transfer of the round spermatid within the ooplasm. The work by Kimura and Yanagimachi[199,203] supports the latter thesis. Fishel et al[202] propose that the genomic imprinting of mouse spermatogenic cells is complete in the testis prior to the male second meiotic division.

The methylation patterns of the alleles of an imprinted gene during male germ development are different. For example, prospermatogonia in the embryo demonstrate an absence of methylation of both the maternal and paternal allele of a maternally expressed gene.[204] The paternal allele of a maternally expressed gene of a prospermatogonium acquires hypermethylation by the 15th day postcoitus. After birth, both parental alleles are fully methylated.

Several investigators have attempted to achieve generation of elongated spermatids or spermatozoa in non-obstructed azoospermic men with early or late maturation arrest.[182] Considering that the imprint establishment in humans has been completed by the time the stage of the round spermatid has been reached,[204] it appears that generation of haploid cells

(from surgically recovered testicular spermatogenic dipoid germ cells) and subsequent ooplasmic injections of the above *in vitro* generated haploid cells represents an attractive mode of treatment for men with early maturation arrest. However, it is unknown whether the rapidly progressing meiosis and early spermiogenesis occurring under conditions present in *in vitro* culture systems allow the completion of the genomic imprinting process within these relative short periods. This is a consideration of clinical importance because abnormalities in the completion of genomic imprinting during *in vitro* spermatogenesis may be manifested after fertilization as tumor susceptibility and/or tumorgenesis.

REFERENCES

1. de Rooij DG, 2009 The spermatogonial stem cell niche. Microsc Res Tech 72: 580-585.
2. Stukenborg JB, Wistuba J, Luetjens CM, et al, 2008 Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. J Androl 29: 312-329.
3. Kanatsu-Shinohara M, Toyokuni S, Morimoto T, Matsui S, Honjo T, Shinohara T, 2003 Functional assessment of self-renewal activity of male germline stem cells following cytotoxic damage and serial transplantation. Biol Reprod 68: 1801-1807.
4. Ogawa T, Ohmura M, Yumura Y, Sawada H, Kubota Y, 2003 Expansion of murine spermatogonial stem cells through serial transplantation. Biol Reprod 68: 316-322.
5. Vergouwen RP, Jacobs SG, Huiskamp R, Davids JA, de Rooij DG, 1991 Proliferative activity of gonocytes, Sertoli cells and interstitial cells during testicular development in mice. J Reprod Fertil 93: 233-243.
6. Kierszenbaum AL, 1994 Mammalian spermatogenesis *in vivo* and *in vitro*: a partnership of spermatogenic and somatic cell lineages. Endocr Rev 15: 116-134.
7. Yamashita YM, Jones DL, Fuller MT, 2003 Orientation of asymmetric stem cell division by the APC tumor suppressor and centrosome. Science 301: 1547-1550.
8. Kiger AA, Jones DL, Schulz C, Rogers MB, Fuller MT, 2001 Stem cell self-renewal specified by JAK-STAT activation in response to a support cell cue. Science 294: 2542-2545.
9. Tulina N, Matunis E, 2001 Control of stem cell self-renewal in Drosophila spermatogenesis by JAK-STAT signaling. Science 294: 2546-2549.
10. Sefton L, Timmer JR, Zhang Y, Beranger F, Cline TW, 2000 An extracellular activator of the Drosophila JAK/STAT pathway is a sex-determination signal element. Nature 405: 970-973.
11. Binari R, Perrimon N, 1994 Stripe-specific regulation of pair-rule genes by hopscotch, a putative Jak family tyrosine kinase in Drosophila. Genes Dev 8: 300-312.
12. Hou XS, Melnick MB, Perrimon N, 1996 Marelle acts downstream of the Drosophila HOP/JAK kinase and encodes a protein similar to the mammalian STATs. Cell 84: 411-419.
13. Aponte PM, van Bragt MP, de Rooij DG, van Pelt AM, 2005 Spermatogonial stem cells: characteristics and experimental possibilities. APMIS 113: 727-742.
14. Clermont Y, 1972 Kinetics of spermatogenesis in mammals: seminiferous epithelium cycle and spermatogonial renewal. Physiol Rev 52: 198-236.
15. Ehmcke J, Schlatt S, 2006 A revised model for spermatogonial expansion in man: lessons from non-human primates. Reproduction 132: 673-680.
16. Ehmcke J, Wistuba J, Schlatt S, 2006 Spermatogonial stem cells: questions, models and perspectives. Hum Reprod Update 12: 275-282.
17. Roosen-Runge EC, Barlow FD, 1953 Quantitative studies on human spermatogenesis. I. Spermatogonia. Am J Anat 93: 143-169.
18. Amann RP, 2008 The cycle of the seminiferous epithelium in humans: a need to revisit? J Androl 29: 469-487.
19. McLachlan RI, O'Donnell L, Meachem SJ, et al, 2002 Identification of specific sites of hormonal regulation in spermatogenesis in rats, monkeys, and man. Recent Prog Horm Res 57: 149-179.
20. Yoshida S, Sukeno M, Nabeshima Y, 2007 A vasculature-associated niche for undifferentiated spermatogonia in the mouse testis. Science 317: 1722-1726.
21. Griswold MD, 1998 The central role of Sertoli cells in spermatogenesis. Semin Cell Dev Biol 9: 411-416.
22. Meachem S, von Schonfeldt V, Schlatt S, 2001 Spermatogonia: stem cells with a great perspective. Reproduction 121: 825-834.
23. Hess RA, Cooke PS, Bunick D, Kirby JD, 1993 Adult testicular enlargement induced by neonatal hypothyroidism is accompanied by increased Sertoli and germ cell numbers. Endocrinology 132: 2607-2613.
24. Orth JM, Gunsalus GL, Lamperti AA, 1988 Evidence from Sertoli cell-depleted rats indicates that spermatid number in adults depends on numbers of Sertoli cells produced during perinatal development. Endocrinology 122: 787-794.
25. Simorangkir DR, de Kretser DM, Wreford NG, 1995 Increased numbers of Sertoli and germ cells in adult rat testes induced by synergistic action of transient neonatal hypothyroidism and neonatal hemicastration. J Reprod Fertil 104: 207-213.
26. Debeljuk L, Rao JN, Bartke A, 2003 Tachykinins and their possible modulatory role on testicular function: a review. Int J Androl 26: 202-210.
27. Allard EK, Blanchard KT, Boekelheide K, 1996 Exog-

enous stem cell factor (SCF) compensates for altered endogenous SCF expression in 2,5-hexanedione-induced testicular atrophy in rats. Biol Reprod 55: 185-193.

28. Blanchard KT, Lee J, Boekelheide K, 1998 Leuprolide, a gonadotropin-releasing hormone agonist, reestablishes spermatogenesis after 2,5-hexanedione-induced irreversible testicular injury in the rat, resulting in normalized stem cell factor expression. Endocrinology 139: 236-244.

29. Marziali G, Lazzaro D, Sorrentino V, 1993 Binding of germ cells to mutant Sld Sertoli cells is defective and is rescued by expression of the transmembrane form of the c-kit ligand. Dev Biol 157: 182-190.

30. Wu JC, Gregory CW, DePhilip RM, 1993 Expression of E-cadherin in immature rat and mouse testis and in rat Sertoli cell cultures. Biol Reprod 49: 1353-1361.

31. Mruk DD, Cheng CY, 2004 Sertoli-Sertoli and Sertoli-germ cell interactions and their significance in germ cell movement in the seminiferous epithelium during spermatogenesis. Endocr Rev 25: 747-806.

32. Erkkila K, Aito H, Aalto K, Pentikainen V, Dunkel L, 2002 Lactate inhibits germ cell apoptosis in the human testis. Mol Hum Reprod 8: 109-117.

33. Riera MF, Meroni SB, Gomez GE, Schteingart HF, Pellizzari EH, Cigorraga SB, 2001 Regulation of lactate production by FSH, iL1beta, and TNFalpha in rat Sertoli cells. Gen Comp Endocrinol 122: 88-97.

34. Riera MF, Meroni SB, Schteingart HF, Pellizzari EH, Cigorraga SB, 2002 Regulation of lactate production and glucose transport as well as of glucose transporter 1 and lactate dehydrogenase A mRNA levels by basic fibroblast growth factor in rat Sertoli cells. J Endocrinol 173: 335-343.

35. Alves MG, Dias TR, Silva BM, Oliveira PF, 2014 Metabolic cooperation in testis as a pharmacological target: from disease to contraception. Curr Mol Pharmacol 7: 83-95.

36. Robinson R, Fritz IB, 1981 Metabolism of glucose by Sertoli cells in culture. Biol Reprod 24: 1032-1041.

37. Oliveira PF, Martins AD, Moreira AC, Cheng CY, Alves MG, 2015 The Warburg effect revisited--lesson from the Sertoli cell. Med Res Rev 35: 126-151.

38. Boussouar F, Benahmed M, 2004 Lactate and energy metabolism in male germ cells. Trends Endocrinol Metab 15: 345-350.

39. Mullaney BP, Rosselli M, Skinner MK, 1994 Developmental regulation of Sertoli cell lactate production by hormones and the testicular paracrine factor, PModS. Mol Cell Endocrinol 104: 67-73.

40. Dias TR, Alves MG, Silva BM, Oliveira PF, 2014 Sperm glucose transport and metabolism in diabetic individuals. Mol Cell Endocrinol 396: 37-45.

41. Rato L, Alves MG, Socorro S, Carvalho RA, Cavaco JE, Oliveira PF, 2012 Metabolic modulation induced by oestradiol and DHT in immature rat Sertoli cells

cultured in vitro. Biosci Rep 32: 61-69.

42. Oliveira PF, Alves MG, Rato L, et al, 2012 Effect of insulin deprivation on metabolism and metabolism-associated gene transcript levels of in vitro cultured human Sertoli cells. Biochim Biophys Acta 1820: 84-89.

43. Oliveira PF, Alves MG, Rato L, et al, 2011 Influence of 5alpha-dihydrotestosterone and 17beta-estradiol on human Sertoli cells metabolism. Int J Androl 34: e612-620.

44. Alves MG, Rato L, Carvalho RA, Moreira PI, Socorro S, Oliveira PF, 2013 Hormonal control of Sertoli cell metabolism regulates spermatogenesis. Cell Mol Life Sci 70: 777-793.

45. Martins AD, Alves MG, Simoes VL, et al, 2013 Control of Sertoli cell metabolism by sex steroid hormones is mediated through modulation in glycolysis-related transporters and enzymes. Cell Tissue Res 354: 861-868.

46. De Gendt K, Swinnen JV, Saunders PT, et al, 2004 A Sertoli cell-selective knockout of the androgen receptor causes spermatogenic arrest in meiosis. Proc Natl Acad Sci U S A 101: 1327-1332.

47. Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA, 2002 Localization of androgen and estrogen receptors in adult male mouse reproductive tract. J Androl 23: 870-881.

48. Holdcraft RW, Braun RE, 2004 Androgen receptor function is required in Sertoli cells for the terminal differentiation of haploid spermatids. Development 131: 459-467.

49. Free MJ, Jaffe RA, 1979 Collection of rete testis fluid from rats without previous efferent duct ligation. Biol Reprod 20: 269-278.

50. Hess RA, 2000 Oestrogen in fluid transport in efferent ducts of the male reproductive tract. Rev Reprod 5: 84-92.

51. Carreau S, Delalande C, Galeraud-Denis I, 2009 Mammalian sperm quality and aromatase expression. Microsc Res Tech 72: 552-557.

52. Li H, Papadopoulos V, Vidic B, Dym M, Culty M, 1997 Regulation of rat testis gonocyte proliferation by platelet-derived growth factor and estradiol: identification of signaling mechanisms involved. Endocrinology 138: 1289-1298.

53. O'Donnell L, Robertson KM, Jones ME, Simpson ER, 2001 Estrogen and spermatogenesis. Endocr Rev 22: 289-318.

54. Cheng CY , Boettcher B, 1979 The effect of steroids on the in vitro migration of washed human spermatozoa in modified Tyrode's solution or in fasting human blood serum. Fertil Steril 32: 566-570.

55. Revelli A, Massobrio M, Tesarik J, 1998 Nongenomic actions of steroid hormones in reproductive tissues. Endocr Rev 19: 3-17.

56. Hicks JJ, Pedron N, Rosado A, 1972 Modifications of

human spermatozoa glycolysis by cyclic adenosine monophosphate (cAMP), estrogens, and follicular fluid. Fertil Steril 23: 886-893.

57. Mishra DP, Shaha C, 2005 Estrogen-induced spermatogenic cell apoptosis occurs via the mitochondrial pathway: role of superoxide and nitric oxide. J Biol Chem 280: 6181-6196.

58. Mruk DD, Cheng CY, 2010 Tight junctions in the testis: new perspectives. Philos Trans R Soc Lond B Biol Sci 365: 1621-1635.

59. Russell L, 1977 Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. Am J Anat 148: 313-328.

60. Fijak M, Bhushan S, Meinhardt A, 2011 Immunoprivileged sites: the testis. Methods Mol Biol 677: 459-470.

61. Setchell BP, Scott TW, Voglmayr JK, Waites GM, 1969 Characteristics of testicular spermatozoa and the fluid which transports them into the epididymis. Biol Reprod 1: Suppl 1: 40-66.

62. Setchell BP, Voglmayr JK, Waites GM, 1969 A blood-testis barrier restricting passage from blood into rete testis fluid but not into lymph. J Physiol 200: 73-85.

63. Alves MG, Martins AD, Cavaco JE, Socorro S, Oliveira PF, 2013 Diabetes, insulin-mediated glucose metabolism and Sertoli/blood-testis barrier function. Tissue Barriers 1: e23992.

64. Rato L, Alves MG, Socorro S, Duarte AI, Cavaco JE, Oliveira PF, 2012 Metabolic regulation is important for spermatogenesis. Nat Rev Urol 9: 330-338.

65. Rebourcet D, O'Shaughnessy PJ, Pitetti JL, et al, 2014 Sertoli cells control peritubular myoid cell fate and support adult Leydig cell development in the prepubertal testis. Development 141: 2139-2149.

66. Kumar TR, Wang Y, Lu N, Matzuk MM, 1997 Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility. Nat Genet 15: 201-204.

67. Tapanainen JS, Aittomaki K, Min J, Vaskivuo T, Huhtaniemi IT, 1997 Men homozygous for an inactivating mutation of the follicle-stimulating hormone (FSH) receptor gene present variable suppression of spermatogenesis and fertility. Nat Genet 15: 205-206.

68. Dierich A, Sairam MR, Monaco L, et al, 1998 Impairing follicle-stimulating hormone (FSH) signaling in vivo: targeted disruption of the FSH receptor leads to aberrant gametogenesis and hormonal imbalance. Proc Natl Acad Sci U S A 95: 13612-13617.

69. Marshall GR, Zorub DS, Plant TM, 1995 Follicle-stimulating hormone amplifies the population of differentiated spermatogonia in the hypophysectomized testosterone-replaced adult rhesus monkey (Macaca mulatta). Endocrinology 136: 3504-3511.

70. Erkkila K, Henriksen K, Hirvonen V, et al, 1997 Testosterone regulates apoptosis in adult human seminiferous tubules *in vitro*. J Clin Endocrinol Metab 82: 2314-2321.

71. Tesarik J, Greco E, Mendoza C, 2001 Assisted reproduction with in-vitro-cultured testicular spermatozoa in cases of severe germ cell apoptosis: a pilot study. Hum Reprod 16: 2640-2645.

72. Lee J, Richburg JH, Younkin SC, Boekelheide K, 1997 The Fas system is a key regulator of germ cell apoptosis in the testis. Endocrinology 138: 2081-2088.

73. Dimitriadis F, Tsambalas S, Tsounapi P, et al, 2010 Effects of phosphodiesterase-5 inhibitors on Leydig cell secretory function in oligoasthenospermic infertile men: a randomized trial. BJU Int 106: 1181-1185.

74. Dimitriadis F, Tsampalas S, Tsounapi P, et al, 2012 Effects of phosphodiesterase-5 inhibitor vardenafil on testicular androgen-binding protein secretion, the maintenance of foci of advanced spermatogenesis and the sperm fertilising capacity in azoospermic men. Andrologia 44: Suppl 1: 144-153.

75. Wayman C, Phillips S, Lunny C, et al, 2005 Phosphodiesterase 11 (PDE11) regulation of spermatozoa physiology. Int J Impot Res 17: 216-223.

76. Melino G, Knight RA, Nicotera P, 2005 How many ways to die? How many different models of cell death? Cell Death Differ 12: Suppl 2: 1457-1462.

77. Lockshin RA, Zakeri Z, 2004 Caspase-independent cell death? Oncogene 23: 2766-2773.

78. Shaha C, 2007 Modulators of spermatogenic cell survival. Soc Reprod Fertil Suppl 63: 173-186.

79. Sinha Hikim AP, Swerdloff RS, 1999 Hormonal and genetic control of germ cell apoptosis in the testis. Rev Reprod 4: 38-47.

80. Show MD, Folmer JS, Anway MD, Zirkin BR, 2004 Testicular expression and distribution of the rat bcl2 modifying factor in response to reduced intratesticular testosterone. Biol Reprod 70: 1153-1161.

81. Mayer F, Honecker F, Looijenga LH, Bokemeyer C, 2003 Towards an understanding of the biological basis of response to cisplatin-based chemotherapy in germ-cell tumors. Ann Oncol 14: 825-832.

82. Hikim AP, Wang C, Lue Y, Johnson L, Wang XH, Swerdloff RS, 1998 Spontaneous germ cell apoptosis in humans: evidence for ethnic differences in the susceptibility of germ cells to programmed cell death. J Clin Endocrinol Metab 83: 152-156.

83. Print CG, Loveland KL, 2000 Germ cell suicide: new insights into apoptosis during spermatogenesis. Bioessays 22: 423-430.

84. Gnessi L, Fabbri A, Spera G, 1997 Gonadal peptides as mediators of development and functional control of the testis: an integrated system with hormones and local environment. Endocr Rev 18: 541-609.

85. Schlatt S, Meinhardt A, Nieschlag E, 1997 Paracrine regulation of cellular interactions in the testis: factors in search of a function. Eur J Endocrinol 137: 107-117.

86. Zou H, Henzel WJ, Liu X, Lutschg A, Wang X, 1997 Apaf-1, a human protein homologous to C. elegans

CED-4, participates in cytochrome c-dependent activation of caspase-3. Cell 90: 405-413.

87. Ross AJ, Waymire KG, Moss JE, et al, 1998 Testicular degeneration in Bclw-deficient mice. Nat Genet 18: 251-256.

88. Yan W, Samson M, Jegou B, Toppari J, 2000 Bcl-w forms complexes with Bax and Bak, and elevated ratios of Bax/Bcl-w and Bak/Bcl-w correspond to spermatogonial and spermatocyte apoptosis in the testis. Mol Endocrinol 14: 682-699.

89. Erkkila K, Pentikainen V, Wikstrom M, Parvinen M, Dunkel L, 1999 Partial oxygen pressure and mitochondrial permeability transition affect germ cell apoptosis in the human testis. J Clin Endocrinol Metab 84: 4253-4259.

90. Adams JM, Cory S, 1998 The Bcl-2 protein family: arbiters of cell survival. Science 281: 1322-1326.

91. Green DR, 2000 Apoptotic pathways: paper wraps stone blunts scissors. Cell 102: 1-4.

92. Oldereid NB, Angelis PD, Wiger R, Clausen OP, 2001 Expression of Bcl-2 family proteins and spontaneous apoptosis in normal human testis. Mol Hum Reprod 7: 403-408.

93. Honarpour N, Du C, Richardson JA, et al, 2000 Adult Apaf-1-deficient mice exhibit male infertility. Dev Biol 218: 248-258.

94. Narisawa S, Hecht NB, Goldberg E, Boatright KM, Reed JC, Millan JL, 2002 Testis-specific cytochrome c-null mice produce functional sperm but undergo early testicular atrophy. Mol Cell Biol 22: 5554-5562.

95. Kimura M, Itoh N, Takagi S, et al, 2003 Balance of apoptosis and proliferation of germ cells related to spermatogenesis in aged men. J Androl 24: 185-191.

96. Watanabe-Fukunaga R, Brannan CI, Itoh N, et al, 1992 The cDNA structure, expression, and chromosomal assignment of the mouse Fas antigen. J Immunol 148: 1274-1279.

97. Suda T, Takahashi T, Golstein P, Nagata S, 1993 Molecular cloning and expression of the Fas ligand, a novel member of the tumor necrosis factor family. Cell 75: 1169-1178.

98. Bellgrau D, Gold D, Selawry H, Moore J, Franzusoff A, Duke RC, 1995 A role for CD95 ligand in preventing graft rejection. Nature 377: 630-632.

99. Sofikitis N, Kaponis A, Mio Y, et al, 2003 Germ cell transplantation: a review and progress report on ICSI from spermatozoa generated in xenogeneic testes. Hum Reprod Update 9: 291-307.

100. Nagata S, Golstein P, 1995 The Fas death factor. Science 267: 1449-1456.

101. Pentikainen V, Erkkila K, Dunkel L, 1999 Fas regulates germ cell apoptosis in the human testis *in vitro*. Am J Physiol 276: E310-316.

102. Sugihara A, Saiki S, Tsuji M, et al, 1997 Expression of Fas and Fas ligand in the testes and testicular germ cell tumors: an immunohistochemical study. Anticancer Res 17: 3861-3865.

103. Francavilla S, D'Abrizio P, Rucci N, et al, 2000 Fas and Fas ligand expression in fetal and adult human testis with normal or deranged spermatogenesis. J Clin Endocrinol Metab 85: 2692-2700.

104. Giebel LB, Strunk KM, Holmes SA, Spritz RA, 1992 Organization and nucleotide sequence of the human KIT (mast/stem cell growth factor receptor) proto-oncogene. Oncogene 7: 2207-2217.

105. Vandenbark GR, deCastro CM, Taylor H, Dew-Knight S, Kaufman RE, 1992 Cloning and structural analysis of the human c-kit gene. Oncogene 7: 1259-1266.

106. Flanagan JG, Chan DC, Leder P, 1991 Transmembrane form of the kit ligand growth factor is determined by alternative splicing and is missing in the Sld mutant. Cell 64: 1025-1035.

107. Manova K, Nocka K, Besmer P, Bachvarova RF, 1990 Gonadal expression of c-kit encoded at the W locus of the mouse. Development 110: 1057-1069.

108. Orth JM, Jester WF Jr., Qiu J, 1996 Gonocytes in testes of neonatal rats express the c-kit gene. Mol Reprod Dev 45: 123-131.

109. Rossi P, Albanesi C, Grimaldi P, Geremia R, 1991 Expression of the mRNA for the ligand of c-kit in mouse Sertoli cells. Biochem Biophys Res Commun 176: 910-914.

110. Mauduit C, Hamamah S, Benahmed M, 1999 Stem cell factor/c-kit system in spermatogenesis. Hum Reprod Update 5: 535-545.

111. Korbutt GS, Elliott JF, Rajotte RV, 1997 Cotransplantation of allogeneic islets with allogeneic testicular cell aggregates allows long-term graft survival without systemic immunosuppression. Diabetes 46: 317-322.

112. Riccioli A, Salvati L, D'Alessio A, et al, 2003 The Fas system in the seminiferous epithelium and its possible extra-testicular role. Andrologia 35: 64-70.

113. Dias TR, Rato L, Martins AD, et al, 2013 Insulin deprivation decreases caspase-dependent apoptotic signaling in cultured rat sertoli cells. ISRN Urol 2013: 970370.

114. Simoes VL, Alves MG, Martins AD, et al, 2013 Regulation of apoptotic signaling pathways by 5alpha-dihydrotestosterone and 17beta-estradiol in immature rat Sertoli cells. J Steroid Biochem Mol Biol 135: 15-23.

115. Cande C, Cohen I, Daugas E, et al, 2002 Apoptosis-inducing factor (AIF): a novel caspase-independent death effector released from mitochondria. Biochimie 84: 215-222.

116. Zou H, Yang R, Hao J, et al, 2003 Regulation of the Apaf-1/caspase-9 apoptosome by caspase-3 and XIAP. J Biol Chem 278: 8091-8098.

117. Page ST, 2011 Physiologic role and regulation of intratesticular sex steroids. Curr Opin Endocrinol

Diabetes Obes 18: 217-223.

118. Lim P, Allan CM, Notini AJ, et al, 2008 Oestradiol-induced spermatogenesis requires a functional androgen receptor. Reprod Fertil Dev 20: 861-870.

119. Andersson AM, Jorgensen N, Frydelund-Larsen L, Rajpert-De Meyts E, Skakkebaek NE, 2004 Impaired Leydig cell function in infertile men: a study of 357 idiopathic infertile men and 318 proven fertile controls. J Clin Endocrinol Metab 89: 3161-3167.

120. Blanco-Rodriguez J, Martinez-Garcia C, 1996 Induction of apoptotic cell death in the seminiferous tubule of the adult rat testis: assessment of the germ cell types that exhibit the ability to enter apoptosis after hormone suppression by oestradiol treatment. Int J Androl 19: 237-247.

121. Pentikainen V, Erkkila K, Suomalainen L, Parvinen M, Dunkel L, 2000 Estradiol acts as a germ cell survival factor in the human testis *in vitro*. J Clin Endocrinol Metab 85: 2057-2067.

122. Sofikitis N, Giotitsas N, Tsounapi P, Baltogiannis D, Giannakis D, Pardalidis N, 2008 Hormonal regulation of spermatogenesis and spermiogenesis. J Steroid Biochem Mol Biol 109: 323-330.

123. Patel YC, Greenwood MT, Panetta R, Demchyshyn L, Niznik H, Srikant CB, 1995 The somatostatin receptor family. Life Sci 57: 1249-1265.

124. Reubi JC, 1997 Relevance of Somatostatin Receptors and Other Peptide Receptors in Pathology. Endocr Pathol 8: 11-20.

125. Vasankari T, Kujala U, Taimela S, Torma A, Irjala K, Huhtaniemi I, 1995 Effects of a long acting somatostatin analog on pituitary, adrenal, and testicular function during rest and acute exercise: unexpected stimulation of testosterone secretion. J Clin Endocrinol Metab 80: 3298-3303.

126. Baou N, Bouras M, Droz JP, Benahmed M, Krantic S, 2000 Evidence for a selective loss of somatostatin receptor subtype expression in male germ cell tumors of seminoma type. Carcinogenesis 21: 805-810.

127. Bruno JF, Xu Y, Song J, Berelowitz M, 1993 Tissue distribution of somatostatin receptor subtype messenger ribonucleic acid in the rat. Endocrinology 133: 2561-2567.

128. Rohrer L, Raulf F, Bruns C, Buettner R, Hofstaedter F, Schule R, 1993 Cloning and characterization of a fourth human somatostatin receptor. Proc Natl Acad Sci U S A 90: 4196-4200.

129. Zhu LJ, Krempels K, Bardin CW, O'Carroll AM, Mezey E, 1998 The localization of messenger ribonucleic acids for somatostatin receptors 1, 2, and 3 in rat testis. Endocrinology 139: 350-357.

130. Goddard I, Bauer S, Gougeon A, et al, 2001 Somatostatin inhibits stem cell factor messenger RNA expression by Sertoli cells and stem cell factor-induced DNA synthesis in isolated seminiferous tubules. Biol

131. Grataroli R, Vindrieux D, Selva J, et al, 2004 Characterization of tumour necrosis factor-alpha-related apoptosis-inducing ligand and its receptors in the adult human testis. Mol Hum Reprod 10: 123-128.

132. Cooke JE, Heasman J, Wylie CC, 1996 The role of interleukin-4 in the regulation of mouse primordial germ cell numbers. Dev Biol 174: 14-21.

133. Dolci S, Williams DE, Ernst MK, et al, 1991 Requirement for mast cell growth factor for primordial germ cell survival in culture. Nature 352: 809-811.

134. Matsui Y, Zsebo K, Hogan BL, 1992 Derivation of pluripotential embryonic stem cells from murine primordial germ cells in culture. Cell 70: 841-847.

135. Baloh RH, Tansey MG, Lampe PA, et al, 1998 Artemin, a novel member of the GDNF ligand family, supports peripheral and central neurons and signals through the GFRalpha3-RET receptor complex. Neuron 21: 1291-1302.

136. Kotzbauer PT, Lampe PA, Heuckeroth RO, et al, 1996 Neurturin, a relative of glial-cell-line-derived neurotrophic factor. Nature 384: 467-470.

137. Lin LF, Doherty DH, Lile JD, Bektesh S, Collins F, 1993 GDNF: a glial cell line-derived neurotrophic factor for midbrain dopaminergic neurons. Science 260: 1130-1132.

138. Milbrandt J, de Sauvage FJ, Fahrner TJ, et al, 1998 Persephin, a novel neurotrophic factor related to GDNF and neurturin. Neuron 20: 245-253.

139. Trupp M, Ryden M, Jornvall H, et al, 1995 Peripheral expression and biological activities of GDNF, a new neurotrophic factor for avian and mammalian peripheral neurons. J Cell Biol 130: 137-148.

140. Meng X, Lindahl M, Hyvonen ME, et al, 2000 Regulation of cell fate decision of undifferentiated spermatogonia by GDNF. Science 287: 1489-1493.

141. Antoniades HN, Hunkapiller MW, 1983 Human platelet-derived growth factor (PDGF): amino-terminal amino acid sequence. Science 220: 963-965.

142. Heldin CH, Westermark B, 1999 Mechanism of action and *in vivo* role of platelet-derived growth factor. Physiol Rev 79: 1283-1316.

143. Basciani S, Mariani S, Arizzi M, et al, 2002 Expression of platelet-derived growth factor-A (PDGF-A), PDGF-B, and PDGF receptor-alpha and -beta during human testicular development and disease. J Clin Endocrinol Metab 87: 2310-2319.

144. Hasthorpe S, Barbic S, Farmer PJ, Hutson JM, 2000 Growth factor and somatic cell regulation of mouse gonocyte-derived colony formation *in vitro*. J Reprod Fertil 119: 85-91.

145. Hasthorpe S, 2003 Clonogenic culture of normal spermatogonia: *in vitro* regulation of postnatal germ cell proliferation. Biol Reprod 68: 1354-1360.

146. Nagano M, Ryu BY, Brinster CJ, Avarbock MR, Brin-

ster RL, 2003 Maintenance of mouse male germ line stem cells *in vitro*. Biol Reprod 68: 2207-2214.

147. Ogawa T, Arechaga JM, Avarbock MR, Brinster RL, 1997 Transplantation of testis germinal cells into mouse seminiferous tubules. Int J Dev Biol 41: 111-122.

148. Meehan T, Schlatt S, O'Bryan MK, de Kretser DM, Loveland KL, 2000 Regulation of germ cell and Sertoli cell development by activin, follistatin, and FSH. Dev Biol 220: 225-237.

149. Toebosch AM, Robertson DM, Trapman J, et al, 1988 Effects of FSH and IGF-I on immature rat Sertoli cells: inhibin alpha- and beta-subunit mRNA levels and inhibin secretion. Mol Cell Endocrinol 55: 101-105.

150. Boitani C, Stefanini M, Fragale A, Morena AR, 1995 Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. Endocrinology 136: 5438-5444.

151. Mather JP, Attie KM, Woodruff TK, Rice GC, Phillips DM, 1990 Activin stimulates spermatogonial proliferation in germ-Sertoli cell cocultures from immature rat testis. Endocrinology 127: 3206-3214.

152. Haneji T, Koide SS, Tajima Y, Nishimune Y, 1991 Differential effects of epidermal growth factor on the differentiation of type A spermatogonia in adult mouse cryptorchid testes *in vitro*. J Endocrinol 128: 383-388.

153. Wahab-Wahlgren A, Martinelle N, Holst M, Jahnukainen K, Parvinen M, Soder O, 2003 EGF stimulates rat spermatogonial DNA synthesis in seminiferous tubule segments *in vitro*. Mol Cell Endocrinol 201: 39-46.

154. Dirami G, Ravindranath N, Pursel V, Dym M, 1999 Effects of stem cell factor and granulocyte macrophage-colony stimulating factor on survival of porcine type A spermatogonia cultured in KSOM. Biol Reprod 61: 225-230.

155. Nakayama Y, Yamamoto T, Abe SI, 1999 IGF-I, IGF-II and insulin promote differentiation of spermatogonia to primary spermatocytes in organ culture of newt testes. Int J Dev Biol 43: 343-347.

156. Tajima Y, Watanabe D, Koshimizu U, Matsuzawa T, Nishimune Y, 1995 Insulin-like growth factor-I and transforming growth factor-alpha stimulate differentiation of type A spermatogonia in organ culture of adult mouse cryptorchid testes. Int J Androl 18: 8-12.

157. Sousa M, Cremades N, Alves C, Silva J, Barros A, 2002 Developmental potential of human spermatogenic cells co-cultured with Sertoli cells. Hum Reprod 17: 161-172.

158. Tesarik J, Greco E, Rienzi L, et al, 1998 Differentiation of spermatogenic cells during *in-vitro* culture of testicular biopsy samples from patients with obstructive azoospermia: effect of recombinant follicle stimulating hormone. Hum Reprod 13: 2772-2781.

159. Tesarik J, Guido M, Mendoza C, Greco E, 1998 Human spermatogenesis *in vitro*: respective effects of follicle-stimulating hormone and testosterone on meiosis, spermiogenesis, and Sertoli cell apoptosis. J Clin Endocrinol Metab 83: 4467-4473.

160. Martin-du Pan RC, Campana A, 1993 Physiopathology of spermatogenic arrest. Fertil Steril 60: 937-946.

161. O'Donnell L, Nicholls PK, O'Bryan MK, McLachlan RI, Stanton PG, 2011 Spermiation: The process of sperm release. Spermatogenesis 1: 14-35.

162. Kerr JB, Millar M, Maddocks S, Sharpe RM, 1993 Stage-dependent changes in spermatogenesis and Sertoli cells in relation to the onset of spermatogenic failure following withdrawal of testosterone. Anat Rec 235: 547-559.

163. O'Donnell L, McLachlan RI, Wreford NG, de Kretser DM, Robertson DM, 1996 Testosterone withdrawal promotes stage-specific detachment of round spermatids from the rat seminiferous epithelium. Biol Reprod 55: 895-901.

164. Sofikitis N, Ono K, Yamamoto Y, Papadopoulos H, Miyagawa I, 1999 Influence of the male reproductive tract on the reproductive potential of round spermatids abnormally released from the seminiferous epithelium. Hum Reprod 14: 1998-2006.

165. Zirkin BR, 1998 Spermatogenesis: its regulation by testosterone and FSH. Semin Cell Dev Biol 9: 417-421.

166. Kim JM, Ghosh SR, Weil AC, Zirkin BR, 2001 Caspase-3 and caspase-activated deoxyribonuclease are associated with testicular germ cell apoptosis resulting from reduced intratesticular testosterone. Endocrinology 142: 3809-3816.

167. Puthalakath H, Villunger A, O'Reilly LA, et al, 2001 Bmf: a proapoptotic BH3-only protein regulated by interaction with the myosin V actin motor complex, activated by anoikis. Science 293: 1829-1832.

168. Russell LD, Weber JE, Vogl AW, 1986 Characterization of filaments within the subacrosomal space of rat spermatids during spermiogenesis. Tissue Cell 18: 887-898.

169. Sluka P, O'Donnell L, Bartles JR, Stanton PG, 2006 FSH regulates the formation of adherens junctions and ectoplasmic specialisations between rat Sertoli cells *in vitro* and *in vivo*. J Endocrinol 189: 381-395.

170. Hannay T, 1995 New Japanese IVF method finally made available in Japan. Nat Med 1: 289-290.

171. Tesarik J, Mendoza C, Testart J, 1995 Viable embryos from injection of round spermatids into oocytes. N Engl J Med 333: 525.

172. Mansour RT, Aboulghar MA, Serour K, 1996 Pregnancy and delivery after intracytoplasmic injection of spermatids into human oocytes. Middle East Fertil. Soc. 1: 223-225.

173. Antinori S, Versaci C, Dani G, Antinori M, Pozza D, Selman HA, 1997 Fertilization with human testicular

spermatids: four successful pregnancies. Hum Reprod 12: 286-291.

174. Vanderzwalmen P, Zech H, Birkenfeld A, et al, 1997 Intracytoplasmic injection of spermatids retrieved from testicular tissue: influence of testicular pathology, type of selected spermatids and oocyte activation. Hum Reprod 12: 1203-1213.

175. Amer M, Soliman E, el-Sadek M, Mendoza C, Tesarik J, 1997 Is complete spermiogenesis failure a good indication for spermatid conception? Lancet 350: 116.

176. Araki Y, Motoyama M, Yoshida A, Kim SY, Sung H, Araki S, 1997 Intracytoplasmic injection with late spermatids: a successful procedure in achieving child-birth for couples in which the male partner suffers from azoospermia due to deficient spermatogenesis. Fertil Steril 67: 559-561.

177. Yamanaka K, Sofikitis NV, Miyagawa I, et al, 1997 Ooplasmic round spermatid nuclear injection proce-dures as an experimental treatment for nonobstructive azoospermia. J Assist Reprod Genet 14: 55-62.

178. Bernabeu R, Cremades N, Takahashi K, Sousa M, 1998 Successful pregnancy after spermatid injection. Hum Reprod 13: 1898-1900.

179. Sofikitis N, Mantzavinos T, Loutradis D, Yamamoto Y, Tarlatzis V, Miyagawa I, 1998 Ooplasmic injec-tions of secondary spermatocytes for non-obstructive azoospermia. Lancet 351: 1177-1178.

180. Sofikitis N, Miyagawa I, Yamamoto Y, Loutradis D, Mantzavinos T, Tarlatzis V, 1998 Micro- and macro-consequences of ooplasmic injections of early haploid male gametes. Hum Reprod Update 4: 197-212.

181. Rajfer J, Turner TT, Rivera F, Howards SS, Sikka SC, 1987 Inhibition of testicular testosterone biosynthesis following experimental varicocele in rats. Biol Reprod 36: 933-937.

182. Sofikitis N, Pappas E, Kawatani A, et al, 2005 Efforts to create an artificial testis: culture systems of male germ cells under biochemical conditions resembling the seminiferous tubular biochemical environment. Hum Reprod Update 11: 229-259.

183. Saito K, O'Donnell L, McLachlan RI, Robertson DM, 2000 Spermiation failure is a major contributor to early spermatogenic suppression caused by hormone withdrawal in adult rats. Endocrinology 141: 2779-2785.

184. Beardsley A, O'Donnell L, 2003 Characterization of normal spermiation and spermiation failure induced by hormone suppression in adult rats. Biol Reprod 68: 1299-1307.

185. Wine RN, Chapin RE, 1999 Adhesion and signaling proteins spatiotemporally associated with spermiation in the rat. J Androl 20: 198-213.

186. McLachlan RI, Wreford NG, de Kretser DM, Robertson DM, 1995 The effects of recombinant follicle-stimu-lating hormone on the restoration of spermatogenesis in the gonadotropin-releasing hormone-immunized adult rat. Endocrinology 136: 4035-4043.

187. Singh J, Handelsman DJ, 1996 The effects of recom-binant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice. J Androl 17: 382-393.

188. Baccetti B, Strehler E, Capitani S, et al, 1997 The ef-fect of follicle stimulating hormone therapy on human sperm structure (Notulae seminologicae 11). Hum Reprod 12: 1955-1968.

189. Krishnamurthy H, Danilovich N, Morales CR, Sairam MR, 2000 Qualitative and quantitative decline in spermatogenesis of the follicle-stimulating hormone receptor knockout (FORKO) mouse. Biol Reprod 62: 1146-1159.

190. Dinulovic D, Radonjic G, 1990 Diabetes mellitus/male infertility. Arch Androl 25: 277-293.

191. Lok S, Johnston DS, Conklin D, et al, 2000 Identifica-tion of INSL6, a new member of the insulin family that is expressed in the testis of the human and rat. Biol Reprod 62: 1593-1599.

192. Jabbour HN, Lincoln GA, 1999 Prolactin receptor expression in the testis of the ram: localisation, func-tional activation and the influence of gonadotrophins. Mol Cell Endocrinol 148: 151-161.

193. Hondo E, Kurohmaru M, Sakai S, Ogawa K, Hayashi Y, 1995 Prolactin receptor expression in rat spermato-genic cells. Biol Reprod 52: 1284-1290.

194. de Winter JP, Themmen AP, Hoogerbrugge JW, Klaij IA, Grootegoed JA, de Jong FH, 1992 Activin recep-tor mRNA expression in rat testicular cell types. Mol Cell Endocrinol 83: R1-8.

195. Andersson AM, Muller J, Skakkebaek NE, 1998 Dif-ferent roles of prepubertal and postpubertal germ cells and Sertoli cells in the regulation of serum inhibin B levels. J Clin Endocrinol Metab 83: 4451-4458.

196. Marchetti C, Hamdane M, Mitchell V, et al, 2003 Immunolocalization of inhibin and activin alpha and betaB subunits and expression of corresponding mes-senger RNAs in the human adult testis. Biol Reprod 68: 230-235.

197. Meinhardt A, O'Bryan MK, McFarlane JR, et al, 1998 Localization of follistatin in the rat testis. J Reprod Fertil 112: 233-241.

198. Tycko B, 1997 Post-graduate course program of The American Society of Andrology. In 22nd Annual Meet-ing of American Society of Andrology. Baltimore, Maryland.

199. Kimura Y, Yanagimachi R, 1995 Mouse oocytes in-jected with testicular spermatozoa or round spermatids can develop into normal offspring. Development 121: 2397-2405.

200. Ogura A, Matsuda J, Yanagimachi R, 1994 Birth of normal young after electrofusion of mouse oocytes with round spermatids. Proc Natl Acad Sci U S A 91:

7460-7462.

201. Sofikitis NV, Miyagawa I, Agapitos E, et al, 1994 Reproductive capacity of the nucleus of the male gamete after completion of meiosis. J Assist Reprod Genet 11: 335-341.

202. Fishel S, Aslam I, Tesarik J, 1996 Spermatid conception: a stage too early, or a time too soon? Hum Reprod 11: 1371-1375.

203. Kimura Y, Yanagimachi R, 1995 Development of normal mice from oocytes injected with secondary spermatocyte nuclei. Biol Reprod 53: 855-862.

204. Georgiou I, Syrrou M, Pardalidis N, et al, 2006 Genetic and epigenetic risks of intracytoplasmic sperm injection method. Asian J Androl 8: 643-673.

205. Swinnen JV, Joseph DR, Conti M, 1989 Molecular cloning of rat homologues of the Drosophila melanogaster dunce cAMP phosphodiesterase: evidence for a family of genes. Proc Natl Acad Sci U S A 86: 5325-5329.

206. Farooqui SM, Al-Bagdadi F, Houslay MD, et al, 2001 Surgically induced cryptorchidism-related degenerative changes in spermatogonia are associated with loss of cyclic adenosine monophosphate-dependent phosphodiesterases type 4 in abdominal testes of rats. Biol Reprod 64: 1583-1589.

207. Levallet G, Levallet J, Bouraima-Lelong H, Bonnamy PJ, 2007 Expression of the cAMP-phosphodiesterase PDE4D isoforms and age-related changes in follicle-stimulating hormone-stimulated PDE4 activities in immature rat sertoli cells. Biol Reprod 76: 794-803.

208. D'Andrea MR, Qiu Y, Haynes-Johnson D, Bhattacharjee S, Kraft P, Lundeen S, 2005 Expression of PDE11A in normal and malignant human tissues. J Histochem Cytochem 53: 895-903.

209. Scipioni A, Stefanini S, Santone R, Giorgi M, 2005 Immunohistochemical localisation of PDE5 in Leydig and myoid cells of prepuberal and adult rat testis. Histochem Cell Biol 124: 401-407.

210. Vasta V, Shimizu-Albergine M, Beavo JA, 2006 Modulation of Leydig cell function by cyclic nucleotide phosphodiesterase 8A. Proc Natl Acad Sci U S A 103: 19925-19930.

211. Baxendale R, Burslem F, Phillips S, 2001 Phosphodiesterase type 11 (PDE11) cellular localization: progress towards defining a physiological role in testis and/or reproduction. J Urol 165: Suppl 5: 340.

212. Degerman E, Belfrage P, Manganiello VC, 1997 Structure, localization, and regulation of cGMP-inhibited phosphodiesterase (PDE3). J Biol Chem 272: 6823-6826.

213. Salanova M, Chun SY, Iona S, Puri C, Stefanini M, Conti M, 1999 Type 4 cyclic adenosine monophosphate-specific phosphodiesterases are expressed in discrete subcellular compartments during rat spermiogenesis. Endocrinology 140: 2297-2306.

214. Yan C, Zhao AZ, Sonnenburg WK, Beavo JA, 2001 Stage and cell-specific expression of calmodulin-dependent phosphodiesterases in mouse testis. Biol Reprod 64: 1746-1754.

215. Fisch JD, Behr B, Conti M, 1998 Enhancement of motility and acrosome reaction in human spermatozoa: differential activation by type-specific phosphodiesterase inhibitors. Hum Reprod 13: 1248-1254.

216. Aversa A, Mazzilli F, Rossi T, Delfino M, Isidori AM, Fabbri A, 2000 Effects of sildenafil (Viagra) administration on seminal parameters and post-ejaculatory refractory time in normal males. Hum Reprod 15: 131-134.

217. Lefievre L, de Lamirande E, Gagnon C, 2002 Presence of cyclic nucleotide phosphodiesterases PDE1A, existing as a stable complex with calmodulin, and PDE3A in human spermatozoa. Biol Reprod 67: 423-430.

218. Baxendale RW, Fraser LR, 2005 Mammalian sperm phosphodiesterases and their involvement in receptor-mediated cell signaling important for capacitation. Mol Reprod Dev 71: 495-508.

219. Clouthier DE, Avarbock MR, Maika SD, Hammer RE, Brinster RL, 1996 Rat spermatogenesis in mouse testis. Nature 381: 418-421.

220. Franca LR, Ogawa T, Avarbock MR, Brinster RL, Russell LD, 1998 Germ cell genotype controls cell cycle during spermatogenesis in the rat. Biol Reprod 59: 1371-1377.

221. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL, 1999 Xenogeneic spermatogenesis following transplantation of hamster germ cells to mouse testes. Biol Reprod 60: 515-521.

222. Sofikitis N, Kaponis A, Giannakopoulos X, Miyagawa I 2004 How far are icsi procedures using human spermatozoa generated into animal testicles? micro- and macro-consequences of germ cell transplantation techniques. In: MG. Colpi (ed) Male infertility today. Fotolito e Stampa Grafiche Gelmini, Milano, Italy; pp, 5-39.